DOWNREGULATION OF ADIPOR1 IS ASSOCIATED WITH INCREASED CIRCULATING ADIPONECTIN LEVELS IN SERBIAN CHRONIC KIDNEY DISEASE PATIENTS

NISHODNA REGULACIJA ADIPOR1 JE UDRUŽENA SA POVIŠENOM KONCENTRACIJOM CIRKULIŠUĆEG ADIPONEKTINA KOD SRPSKIH PACIJENATA SA HRONIČNOM BUBREŽNOM INSUFICIJENCIJOM

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Summary

Background: Since the rise in plasma adiponectin levels in chronic kidney disease (CKD) patients has not yet been elucidated, we sought to investigate if patients on hemodialysis (HD) have altered expression of adiponectin receptors in peripheral blood mononuclear cells (PBMCs) compared to healthy subjects.
Methods: This study included 31 patients with chronic kidney disease on HD and 33 healthy subjects (CG). Circulating adiponectin levels were measured by ELISA while AdipoR1 and AdipoR2 mRNA levels in PBMCs were determined by real-time PCR.
Results: Plasma adiponectin levels were significantly higher in patients compared to control group (P=0.036). After adjustment for age, BMI and creatinine, this difference became even more significant (P=0.004). In both groups adiponectin correlated with creatinine (CG: r=-0.472, P=0.006; HD: r=-0.375, P=0.038), triglycerides (CG: r=-0.490, P=0.004; HD: r=-0.488, P=0.005), insulin (CG: r=-0.386, P=0.038; HD: r=-0.506, P=0.012) and high density lipoprotein cholesterol (HDL-C) (CG: r=-0.672, P<0.001; HD: r=-0.584, P=0.001). Significantly lower expression of PBMCs AdipoR1 mRNA was found in patients compared to CG (P=0.034), while AdipoR2 mRNA levels were similarly expressed in PBMCs in both groups.
Conclusions: Complex pathological processes in CKD cause downregulation of AdipoR1 which could ultimately influence AdipoR1 protein levels leading to a state of »adiponectin resistance«.

Keywords: adiponectin, AdipoR1, AdipoR2, chronic kidney disease

Kratak sadržaj

Uvod: S obzirom na to da razlozi povišenih vrednosti adiponektina u hroničnoj bubrežnoj insuficijenciji (HBI) još uvek nisu razjašnjeni, cilj ovog istraživanja bio je da se ispita da li pacijenti na hemodijalizi (HD) imaju promenjen nivo genske ekspresije adiponektinskih receptora u mononuklearnim ćelijama periferne krvi u odnosu na zdrave ispitanike.
Metode: Ova studija je obuhvatila 31 pacijenta sa hroničnom bubrežnom insuficijencijom na hemodijalizi (HD) i 33 zdrava ispitanika (KG). Koncentracija cirkulišućeg adiponektina određena je ELISA metodom, a nivoi iRNK adiponektinskih receptora AdipoR1 i AdipoR2 u mononuklearnim ćelijama periferne krvi određeni su metodom *real-time PCR*.
Rezultati: Koncentracija adiponektina u plazmi pacijenata bila je značajno viša u odnosu na zdrave ispitanike (P=0,036). Nakon korekcije za godine, BMI i koncentraciju kreatinina, ova razlika je postala još značajnija (P=0,004). U obe grupe ispitanika koncentracija adiponektina je korelisala sa koncentracijom kreatinina (KG: r=-0,472, P=0,006; HD: r=-0,375, P=0,038), koncentracijom triglicerida (KG: r=-0,490, P=0,004; HD: r=-0,488, P=0,005), koncentracijom insulina (KG: r=-0,386, P=0,038; HD: r=-0,506, P=0,012) i koncentracijom holesterola u lipoproteinima velike gustine (KG: r=-0,672, P<0,001; HD: r=-0,584, P=0,001). Pacijenti su imali značajno niži nivo iRNK AdipoR1 u mononuklearnim ćelijama periferne krvi u poređenju sa zdravim ispitanicima (P=0,034), dok se nivo iRNK AdipoR2 nije značajno razlikovao među posmatranim grupama.
Zaključak: Kompleksni patološki procesi u HBI izazivaju nishodnu regulaciju AdipoR1 gena, što bi moglo uticati na nivo AdipoR1 proteina, vodeći u stanje »adiponektinske rezistencije«.

Ključne reči: adiponektin, AdipoR1, AdipoR2, hronična bubrežna insuficijencija

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Introduction

Adiponectin is a protein abundantly secreted by adipose tissue (1). It is generally considered a molecule with antiinflammatory, atheroprotective and antiapoptotic effects which are mainly achieved via activation of two ubiquitously expressed adiponectin receptors, AdipoR1 and AdipoR2 (2). Activation of AdipoR1 leads to increased sensitivity of peripheral tissues to insulin, while AdipoR2 activation increases fatty acid combustion (1, 2). Although the mechanism is not well understood, it has been shown that adiponectin via these two receptors also activates ceramidase which is important for its antiapoptotic effects (3).

Peripheral blood mononuclear cells (PBMC) and macrophages express both AdipoR1 and AdipoR2 allowing adiponectin to affect directly their function. Under this influence, macrophages increase IL-10 secretion and shift from the proinflammatory M1 to the antiinflammatory M2 phenotype (4, 5). It has also been shown that adiponectin exerts protective effects on kidneys by decreasing podocytes albumin permeability in mice (6). It seems that adiponectin downregulates podocytes NADPH oxidase production via AdipoR1 thus decreasing oxidative stress in these cells (7). Martinez et al. suggested that increased oxidative stress together with the altered gene expression of adiponectin receptors could be related to increased insulin resistance in patients with end-stage renal kidney disease (18).

Patients with chronic kidney disease (CKD) are greatly exposed to high risk of cardiovascular disease (CVD) mortality due to the presence of several traditional and nontraditional risk factors (8, 9). Previous studies consistently reported that adiponectin levels are increased in patients with CKD, but the reasons for this phenomenon have remained unclear. Possible explanations include metabolic changes in uremia, reduced adiponectin clearance by kidneys and changes in ligand/receptor reactivity (10–12). Although it was suggested that kidneys play an important role in adiponectin clearance, it has been shown that in successful renal transplantation adiponectin levels do decrease, but still remain higher compared to healthy individuals (13). On the other hand, Halberg et al. have demonstrated in their study that the main site of adiponectin clearance is the liver, and that kidneys only eliminate final degradation products (14). Despite the generally known protective effects of adiponectin, its role in CKD seems to be controversial. While several meta-analyses have linked higher adiponectin levels in CKD patients with lower risk of CVD mortality (15, 16), others have concluded that CVD mortality in CKD patients is associated with increased adiponectin levels (8).

Although numerous studies have indicated that increased circulating adiponectin is indisputably associated with CKD, the reasons for this rise still remain unclear. Since impairment of the adiponectin/receptor axis could contribute to the adiponectin rise, we sought to investigate if patients on hemodialysis (HD) have an altered expression of adiponectin receptors in PBMCs compared to healthy subjects.

Materials and Methods

Subjects

Thirty-one patients (19 males and 12 females; age range 27–76 years, age median 58 years) with CKD on hemodialysis were recruited from the Nephrology Clinic, Clinical Center of Serbia. The main causes of CKD in these patients were: chronic glomerulonephritis (n=6), chronic renal insufficiency (n=4), nephroangiosclerosis (n=6), uric acid nephropathy (n=2), Good-Pasture syndrome (n=2), renal calculosis (n=2), vesicoureteral reflux (n=2), hypertension (n=1), atrophia renis segmentalis (n=1), lupus nephritis (n=1), endemic nephropathy (n=1), chronic pyelonephritis (n=1), renal tuberculosis (n=1), renal vasculitis (n=1). The duration of hemodialysis ranged from 3 to 248 days, with an average of 90 days.

Thirty-three healthy subjects (15 males and 18 females; age range 27–60, age median 45) were selected for the control group. They were interviewed about their family history and health status. Inclusion criteria for the control group were absence of cardiovascular diseases in family history; absence of hypertension (diastolic blood pressure < 90 mm Hg and/or systolic blood pressure < 140 mm Hg) and/or absence of any antihypertensive therapy; favorable lipid profile (according to Adult Treatment Panel III guidelines – ATP III); glucose < 6.1 mmol/L; IMT in at least one carotid artery < 1 mm.

The study was conducted according to the guidelines laid down in the Declaration of Helsinki. All the procedures involving human subjects were approved by the Ethical Committee of the Faculty of Pharmacy, University of Belgrade and School of Medicine, University of Belgrade. All the participants have signed an informed consent before the enrolment.

Samples

Blood samples were collected after a 12-hour fasting period into EDTA-containing tubes. Whole blood was centrifuged for 10 min at 3000 rpm; plasma was separated, frozen and stored at –80 °C until analysis. PBMCs from whole blood were isolated using Ficoll-Paque® gradient and immediately added to TRIzol™ extraction (Invitrogen Life Technologies, Carlsbad, CA, USA) (TRI) reagent and then stored –80 °C until RNA isolation.

Analytical methods

Glucose, total cholesterol (TC), HDL-cholesterol (HDL-C), LDL-cholesterol (LDL-C), triglycerides (TG), urea, creatinine were measured by routine enzymatic methods in plasma using an ILab 300+ analyzer (Instrumentation Laboratory, Milan, Italy) and Randox Laboratories (Armdore, UK) reagents. Fasting insulin concentration was measured by the ECLIA method, on a COBAS®INTEGRA 400 plus – Roche Diagnostics.

In the plasma of all subjects, circulating adiponectin was measured using a Human Total Adiponectin/Acrp30 Quantikine ELISA Kit. Sensitivity of this assay was 0.891 ng/mL, intra-assay and inter-assay coefficients of variation were 3.4% and 5.8% respectively. All the samples were measured in duplicates.

Methods used for RNA isolation, reverse transcription and real-time PCR were described in detail elsewhere (17). Briefly, RNA was isolated using a TRIzol™ extraction (Invitrogen Life Technologies, Carlsbad, CA, USA) (TRI) reagent according to manufacturer's protocol and quantified by absorbance spectrometry at 260 nm. In order to estimate organic and protein contamination, in every sample absorbance was measured at 230 nm and 280 nm, respectively. RNA integrity was evaluated by electrophoresis on 1% agarose gel. Reverse transcription and real-time PCR experiments were performed on the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using TaqMan® reagent-based chemistry. Data were expressed as a ratio between the target gene mRNA level and the mRNA level of the housekeeping gene (beta-actin).

Statistical analysis

Statistical analysis was performed using IBM® SPSS® Statistics version 22 software. Distribution of data was tested with the Shapiro-Wilk test. Normally distributed data are presented as mean ± standard deviation. For the non-normally distributed data logarithmic transformation was performed and these data are presented as geometrical means and 95% confidence intervals. If data could not be transformed to fit normal distribution they are presented as medians and interquartile ranges. Statistical tests used for normally distributed data were: Pearson's correlation, independent samples t-test and ANOVA. Adjustments were performed by using the analysis of covariance (ANCOVA). For the data that had not been normally distributed, Mann-Whitney U test and Spearman's correlation were used. Chi-square test was used for the comparison of categorical variables. Statistically significant p-value was less than 0.05.

Results

Anthropometric and biochemical data of healthy subjects and patients on HD are presented in *Table I*. Levels of urea, creatinine, TG, HDL-C and hsCRP as well as IMT were increased in HD patients compared to healthy subjects (P<0.001, P<0.001, P=0.006, P<0.001, P<0.001, P<0.001, respectively). Levels of insulin were also significantly higher in patients compared to control subjects (P<0.001). On the contrary, BMI and TC were significantly higher in the control group (P=0.001, P=0.047, respectively), while LDL-C levels showed no difference between the groups (P=0.084).

Table I Basic anthropometric, lipid status, inflammation parameters in healthy children according to the age subgroups.

	CG	HD	P
Male/female	15/18	19/12	0.132***
Age (years)	45±9	55±15	0.003*
BMI (kg/m^2)	24.9±3.6	22.2±3.0	0.002*
Urea (mmol/L)	5.76 (5.33–6.23)	21.16 (19.60–22.84)	<0.001*
Creatinine (μmol/L)	86.5 (82.7–90.4)	982.4 (929.4–1038.4)	<0.001*
TC (mmol/L)	5.07±0.92	4.60±0.94	0.047*
HDL-C (mmol/L)	1.60 (1.47–1.75)	0.85 (0.76–0.95)	<0.001*
LDL-C (mmol/L)	3.09±0.76	2.73±0.83	0.084*
TG (mmol/L)	1.24 (1.06–1.47)	1.75 (1.46–2.11)	0.006*
hsCRP (mg/L)	1.16 (0.71–1.45)	5.20 (2.62–11.17)	<0.001**
Insulin (μU/L)	8.90±3.48	42.29±39.91	<0.001*
Adiponectin (μg/mL)	9.60 (7.64–12.04)	13.42 (10.72–16.80)	0.036*
IMT (mm)	0.45 (0.00–0.50)	0.65 (0.55–0.70)	<0.001**

Urea, Creatinine, HDL-C, TG, adiponectin and IMT are logarithmically transformed before statistical analysis and presented as geometrical mean and 95% confidence intervals (CI). Age, BMI, TC and LDL-C are presented as mean ± standard deviation.
*Variables were compared by Student's t-test.
**Variables were compared by Mann-Whitney U test.
***Variables were compared by Chi-square test.
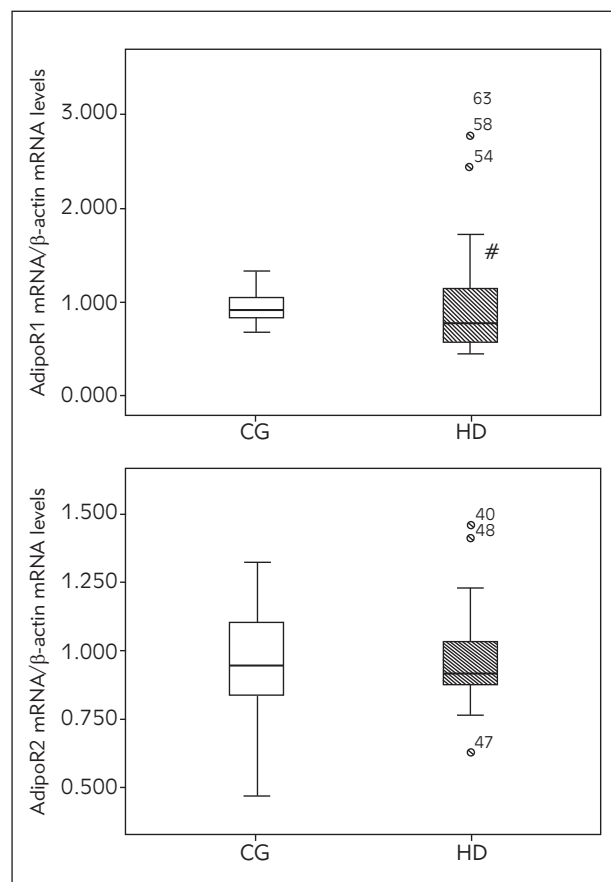
Figure 1 Normalized AdipoR1 (A) and Adipor2 (B) mRNA levels from PBMCs of healthy subjects (CG) and CKD patients on hemodialysis (HD).

– significantly different compared to healthy subjects; P=0.034.

Plasma adiponectin levels were significantly higher in patients compared to control group (P=0.036, *Table I*). After adjustment for age, BMI and creatinine, this difference became even more significant (P=0.004). Males had significantly lower adiponectin levels than females in the control group (6.26 (4.76–8.22) μg/mL vs. 13.70 (10.55–17.80) μg/mL, p<0.001) as well as in the patient group (10.54 (8.17–13.60) μg/mL vs. 19.84 (14.79–26.63) μg/mL, P=0.004). After adjustment for BMI these differences remained significant (P=0.009, P=0.003, respectively).

Furthermore, significantly lower expression of PBMCs AdipoR1 mRNA was found in patients compared to healthy subjects (P=0.034), while AdipoR2 mRNA levels were similarly expressed in PBMCs in both groups (*Figure 1*). We have found a strong correlation between adiponectin receptors in CG (r= 0.666, P<0.001). However, correlation between the two adiponectin receptors was not demonstrated in the patient group (r=-0.061, P=0.757). We have also demonstrated a significant negative correlation between AdipoR1 mRNA levels and insulin concentration, but only in the patient group (r=-0.469, P=0.021).

Correlations of adiponectin with other parameters, in healthy subjects and patients with CKD, are presented in *Table II*. In both groups, adiponectin negatively correlated with creatinine, insulin, TG, and positively with HDL-C. Negative correlation was observed between adiponectin and BMI only in the control group.

Table II Correlation of adiponectin and different parameters in healthy subjects (CG) and CKD patients on hemodialysis (HD).

	Adiponectin (μg/mL)			
	CG		HD	
	r	P	r	P
Age (years)	0.059	0.743	0.123	0.509
BMI (kg/m^2)	-0.613	<0.001	-0.193	0.317
Urea (mmol/L)	0.028	0.879	0.222	0.229
Creatinine (μmol/L)	-0.472	0.006	-0.375	0.038
TC (mmol/L)	0.120	0.505	0.070	0.707
HDL-C (mmol/L)	0.672	<0.001	0.584	0.001
LDL-C (mmol/L)	0.192	0.293	0.157	0.415
TG (mmol/L)	-0.490	0.004	-0.488	0.005
hsCRP (mg/L)	-0.305	0.084	-0.356	0.074
Insulin (μU/L)	-0.386	0.038	-0.506	0.012
IMT (mm)	0.162	0.483	0.105	0.561
AdipoR1*	-0.041	0.822	0.131	0.481
AdipoR2*	-0.131	0.468	-0.177	0.368

*parameters were tested by Spearman's correlation analysis.

Discussion

In this study, we have shown that downregulation of AdipoR1 mRNA in PBMCs (*Figure 1*) and increase in circulating adiponectin levels are present in CKD patients on hemodialysis (*Table I*). Patients were significantly older than controls, but since age did not significantly correlate with either adiponectin or adiponectin receptors, we feel that the obtained results were not influenced by the age difference between the observed subjects.

Increased adiponectin levels in patients on hemodialysis were demonstrated in numerous studies over the past decade, but the reason(s) for this rise have remained unclear. Although kidneys play an important role in the elimination of adiponectin, its elevated levels cannot solely be linked to the deterioration of their function (18). It has been shown that urinary adiponectin levels are also increased in patients with CKD and that after successful kidney transplantation adiponectin levels do decrease, but still remain higher compared to healthy individuals (13). Overproduction and secretion of adiponectin by adipose tissue could be an important contributor to its increase in circulation, but data regarding this subject are not consistent and require further clarifications. While Martinez et al. reported increased expression of both adiponectin mRNA and protein in visceral and subcutaneous fat tissue in patients with end stage kidney disease (19), Marchlewska et al. (20) demonstrated opposite results.

Another mechanism that could contribute to the increase of adiponectin levels in HD patients is the loss of its effects due to downregulation of its receptors (21), which has already been demonstrated in patients with chronic heart failure (CHF) (22) and coronary artery disease (23). In our study, we have found that patients on hemodialysis have decreased expression of AdipoR1 mRNA in PBMCs compared to healthy subjects (*Figure 1*). This implies that various pathological processes in chronic kidney disease cause downregulation of AdipoR1. Long term decrease of AdipoR1 mRNA levels could ultimately lead to decrease of AdipoR1 protein levels on cell surface creating a possibility for the development of »adiponectin resistance«.

Initial stimuli for this downregulation are still unclear. Previous studies have suggested that increased insulin levels are associated with CKD, which was confirmed by our study. We have also found a significant negative correlation between AdipoR1 mRNA levels and insulin concentration but only in patients which suggests that the observed AdipoR1 downregulation could be partly attributed to insulin. Previous studies have also underlined the influence of insulin on AdipoR1 expression. Namely, it has been shown that insulin leads to downregulation of AdipoR1 expression, through the activation of phosphoinositide 3-kinase (PI3-kinase)/Foxo1 pathway (21). Moreover, a strong correlation between the two adiponectin receptors was demonstrated only in CG, while in patients on HD this connection was lost. Hence, the downregulation of AdipoR1 could be a consequence of the pathological processes that act solely upon the regulation of the expression of AdipoR1, but not AdipoR2.

Our results are not in line with two previous studies in which CKD patients had elevated expression of adiponectin receptors (19, 24). These discrepancies could be a consequence of several factors. Patients in previous studies had higher BMI (26.0 ± 5.0 and 27.3 ± 4.4 kg/m^2) than patients in our study (22.2 ± 3.0 kg/m^2). In line with this, Tsigalou et al. showed that beneficial effects of high adiponectin levels on survival were observed only in a subgroup of HD patients with high BMI (>24 kg/m^2) which could be due to the upregulation of adiponectin receptors in CKD patients with higher BMI (25). Also, subjects in previous studies had been on hemodialysis for a much longer period of time (average: 3.5 years) than our patients (average: 90 days). And finally, in the study by Martinez et al. (19) patients that had kidney transplantation as well as patients with diabetes mellitus type two were included, while in our study those subjects were excluded.

We have also shown that adiponectin is related to lipid parameters in both groups, negatively with TG and positively with HDL-C (*Table II*). Two previous studies have shown that despite the fact that adiponectin was positively associated with HDL-C, high rather than low adiponectin was related to increased all-cause mortality in CKD patients (17) and increased all-cause mortality and progression to end stage renal disease (ESKD) in type I diabetic patients (26). Namely, patients with end stage kidney disease often have altered HDL particles composition, leading to impaired functions of HDL and loss of its antiatherogenic properties (27, 28) which is associated with increased CVD risk (29).

It should be noted that the results presented in this study have certain limitations. Firstly, generalization of our conclusion is limited by the small sample size and low statistical power, so a case-control study including more subjects is needed to further elucidate the relation between adiponectin and the development of CKD. Secondly, various causes led to kidney failure in these patients which could have also influenced our results. Thirdly, we have only measured mRNA levels of adiponectin receptors, but not their protein levels which could have strengthened our findings of the receptors downregulation.

In summary, our study has demonstrated downregulation of AdipoR1 and higher levels of circulating adiponectin in CKD patients on hemodialysis. It seems that, in such a complex milieu as one created

during CKD, AdipoR1 is downregulated, which in the long term could reduce AdipoR1 protein levels thus leading to a state of »adiponectin resistance«.

Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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