Proteomic Analysis Reveals the Association between the Rho-GDI Signaling Pathway and Kidney Injury in Spontaneously Hypertensive and Hyperlipidemic Rats

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Abstract

Chronic kidney disease (CKD) is characterized by progressive, irreversible kidney damage, and the number of patients with CKD has been increasing worldwide. Several studies suggested that hypertension, dyslipidemia, and diabetes are risk factors for CKD. In this study, we generated spontaneously hypertensive and hyperlipidemic rats (SHHRs). In SHHRs, total cholesterol and plasma glucose levels become elevated when fed a high-fat, 30% sucrose diet (HFDS). However, the molecular mechanism underlying the effect of hypertensive, dyslipidemic, and hyperglycemic conditions on the kidney remains unknown. To elucidate the mechanism, we performed proteomic analysis of the kidney in SHHRs. Four-month-old male Sprague-Dawley (SD) rats and SHHRs were fed a normal diet (ND) or HFDS *ad libitum* for 6 months. Proteins in the renal cytoplasm were separated by LC/MS, and proteomic analysis was performed. Differentially expressed proteins were linked using Ingenuity Pathway Analysis (IPA). We identified Rho-GDP dissociation inhibitor (Rho-GDI) signaling as a candidate pathway involved in kidney injury in SHHRs with HFDS feeding. In the kidney, the levels of Rho-GDI protein and Rho GTPase-activating protein (Rho-GAP) decreased, and that of p21-activated kinase (PAK) significantly increased. In addition, the renal expression of *Arhgdia* (encoding Rho-GDI), *Dlc1* (encoding Rho-GAP), and *Pak1* (encoding PAK) genes were, at least in part, parallel to that of proteins in SHHRs that had been fed the HFDS. Furthermore, neutrophil gelatinase-associated lipocalin (*Ngal*), a biomarker of kidney injury, was highly expressed in the kidney of SHHRs that had been fed the HFDS. These data suggest the possibility that the Rho-GDI signaling pathway is activated in the kidney of SHHRs that are fed the HFDS, leading to kidney injury. Taken together, our findings provide a molecular basis for the effects of hypertensive, dyslipidemic, and hyperglycemic conditions on the kidney.

Key words

kidney, proteomic analysis, SHHR, Rho-GDI signaling

Introduction

Recently, the number of patients with chronic kidney disease (CKD) has increased, reaching 13.3 million in Japan in 2005¹⁾. It is well known that CKD

directly increases mortality and decreases the ability to perform activities of daily living. Hypertension, dyslipidemia, and diabetes are all risk factors for CKD pathogenesis^{2–4)}. Hypertension leads to sclerosis of the afferent arterioles and causes a loss of autore-

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gulation in the glomerulus⁵). Hypertension also causes arteriolar lumen narrowing, resulting in decreased blood flow in the glomerulus and tubulointerstitium⁵). In addition, the deposition of excessive low-density lipoprotein cholesterol can activate glomerular macrophages⁶). These macrophages produce reactive oxygen species and damage vascular endothelial cells and mesangial cells via inflammatory and fibrogenic factors. Furthermore, the increased concentration of intracellular glucose activates a polyol pathway of glucose metabolism, causing kidney injury through the accumulation of advanced glycation end-products and the activation of oxidative stress⁷).

We previously established a spontaneously hypertensive hyperlipidemic rat (SHHR) line by crossing Sprague–Dawley (SD)-derived hyperlipidemic rats with Wistar–Kyoto-derived spontaneously hypertensive rats (SHRs)⁸). SHHRs have systolic blood pressure higher than 150 mmHg and a total cholesterol level greater than 150 mg/dl when receiving a normal diet. Furthermore, SHHRs experience elevated total cholesterol and plasma glucose levels when fed a high-fat, 30% sucrose diet (HFDS)⁹). Therefore, this model is useful for studying physiology, pathogenesis, and pharmacology under hypertensive, hyperlipidemic, and hyperglycemic conditions. Using this rat model, we previously found that visceral fat accumulates in the body and that plasma levels of oxidative stress markers increase in SHHRs fed with HFDS^{9, 10}). In addition, Oda et al. showed that the protein expression of glutathione peroxidase, which functions as an antioxidant, decreases in the kidney of SHHRs that are fed the HFDS¹¹). These data suggest that oxidative stress accumulates in the kidneys of SHHRs that are fed the HFDS, which could cause kidney injury. However, the molecular mechanism underlying the effects of the hypertensive, hyperlipidemic, and hyperglycemic conditions on the kidney remains unknown. In the present study, we performed proteomic analysis to investigate which molecules in the kidney are involved in hypertension, hyperlipidemia, and hyperglycemia.

Materials and Methods

Animals

We used 10-month-old male SD rats and SHHRs for experiments. The rats were housed in a semi-barrier system with controlled room temperature ($23 \pm 1^\circ\text{C}$), humidity ($55 \pm 5\%$), and lighting (lights on from 6:00 to 18:00). Until the age of 3 months, all rats were fed the normal diet (ND) *ad libitum*. The

SD rats and SHHRs were then divided into 2 groups each. One group was fed the ND for 7 months, and the other group was orally administered 100 mg/L of N^G-nitro-L-arginine methyl ester in drinking water for 1 month and fed the HFDS for 6 months. The ND was regular rat chow (CE2; CLEA Japan Inc., Tokyo, Japan) comprised of 8.9% water, 25.4% protein, 4.4% fat, 4.1% fiber, 6.9% carbohydrate, and 50.3% nitrogen-free extracts, containing 342.2 kcal/100 g. The high-fat diet consisted of 8.2% water, 23.4% protein, 11.0% fat, 3.8% fiber, 6.3% carbohydrate, and 46.3% nitrogen-free extracts, containing 378.0 kcal/100 g (CLEA, Japan). After 6-month feeding on the respective diets, the rats were euthanized under pentobarbital anesthesia, and the weight of visceral adipose and epididymal adipose tissue was determined. Serum was obtained from blood samples by centrifugation and used for the measurement of the derivatives of reactive oxygen metabolite (d-ROM) concentrations using the Free Radical Analytical System (Diacron International, Grosseto, Italy). All experiments were conducted according to the Guiding Principles for the Care and Use of Laboratory Animals of St. Marianna University School of Medicine.

Extraction of subcellular proteomes

Cytosolic and membrane/organelle protein fractions of tissues were isolated from rat kidneys using the ProteoExtract Subcellular Proteome Extraction Kit (Millipore, Billerica, MA, USA) following the manufacturer's procedure. In brief, 50 mg of fragmented kidney was homogenized in 100 μL of Extraction Buffer I with Protease Inhibitor Cocktail (Millipore), and incubated for 10 min at 4°C with shaking. The supernatant was separated by centrifugation for 10 min at $1000 \times g$ at 4°C (as the cytosolic protein fraction). The pellet was resuspended in 300 μL of Extraction Buffer II with Protease Inhibitor Cocktail (Millipore), and incubated for 30 min at 4°C with shaking. The supernatant was separated by centrifugation for 10 min at $6000 \times g$ at 4°C (as the membrane/organelle protein fraction).

Nano-HPLC/ESI-ion trap-MS/MS analysis

The supernatants (5 μL) obtained from the kidney samples were diluted and analyzed using a nano-HPLC system (1200 Series; Agilent Technologies Inc., Palo Alto, CA, USA), connected to an HCT Ultra ion trap mass spectrometer (Bruker Daltonics, Billerica, MA, USA). A 5CD-C18 trap column (Imtakt, Kyoto, Japan) was used to concentrate and de-

salt the injected samples. Chromatographic separation of peptides was carried out using a 5CD-C18 analytical column. Sample elution through the analytical column was obtained at a flow rate of 300 nL/min by starting the following gradient: 5–40% solvent B for 100 min; step with 80% solvent B for 5 min; and step with 5% solvent B for 15 min. Solvent was 0.1% formic acid in water, and solvent B was 0.1% formic acid in acetonitrile. The HCT Ultra was operated in positive-ion mode with a maximum accumulation time of 200 ms in an *m/z* range of 350–1300. The auto-MS/MS analysis was carried out with 3 precursor ions, an absolute threshold of 1000, and an active ion precursor exclusion for 0.5 min after two MS/MS spectra were obtained. The peptides were fragmented in the CID mode. Fragmentation parameters were as follows: width 4.0 *m/z*; and time of fragmentation 200 ms. The mass spectrometry data obtained were analyzed using MASCOT software (Matrix Science, London, UK).

Ingenuity Pathway Analysis

Proteomic analysis data were analyzed with Ingenuity Pathway Analysis (IPA) software following the manufacturer's procedure (<http://www.ingenuity.com/>). The significance of the common networks and canonical pathways were tested based on the *p*-value. The associative networks were shown based on the score.

Quantitative RT-PCR analysis

Total RNA was extracted from the specimens using the RNeasy Mini Kit (Qiagen, Frederick, MD, USA), following the manufacturer's instructions. A RETROscript Kit (Ambion, Austin, TX, USA) was used to synthesize cDNA from total RNA. cDNA was amplified with the LightCycler SYBR Green Master mix using a LightCycler (Roche Diagnostics, Mannheim, Germany). Fluorescence data were analyzed with LightCycler software (Roche Diagnostics). Expressions of target genes were normalized to *Gapdh* mRNA levels measured concurrently. The primer list is given in **Table 1**.

Statistical analysis

For IPA, the *p*-value was calculated using Fisher's exact test. The significance of differences among groups was determined by the analysis of variance (ANOVA) Tukey-Kramer post hoc test. A *p*-value of 0.05 was considered to represent a statistically significant difference.

Table 1. Primer Pairs for RT-PCR Analysis.

Gene		Primer sequence
<i>Arhgdia</i>	Forward	CAATGTCCCCAACGTGATTGTGACC
	Reverse	TTATCCGGTACTCCACACCTTCCTT
<i>Dlc1</i>	Forward	AGTTTGGGCAAACAGACCA
	Reverse	GGCTTCAGTCTCTGTTCACT
<i>Pak1</i>	Forward	ATGCTTTGACCCGGAATACT
	Reverse	TGAAGCACCTTGCCAATCT
<i>Ngal</i>	Forward	ACTCAACTCAGAACTTGATCCCTGC
	Reverse	AAGCGGCTTTGTCTTTCTTTCTGGA
<i>Gapdh</i>	Forward	CTGAGTATGTCGTGGAGTCTA
	Reverse	CTGCTTCAACACCTTCTTGAT

Results

Biological studies of SD rats and SHHRs.

Body weight and kidney weight did not change in either SD rats or SHHRs (**Fig. 1A, 1B**). Visceral adipose tissue did not increase in SD rats that were fed the HFDS (SD-HFDS group), while it was significantly increased in SHHRs fed the same diet (SHHR-HFDS group) (**Fig. 1C**). The amount of epididymal adipose tissue did not change in either SD rats or SHHRs on the HFDS (**Fig. 1D**). The oxidative stress marker d-ROM significantly increased in serum in the HFDS groups, although there was no difference between SD rats and SHHRs (**Fig. 1E**).

Proteomic analysis identified Rho-GDI signaling in the kidney of SHHRs.

When proteomic analysis and IPA were performed to investigate the effects of hypertension, dyslipidemia, and hyperglycemia on the SHHR kidney, the results showed that the level of Rho-GDI protein decreased, and that of p21-activated kinase (PAK) increased in the kidney of SD-HFDS compared with SD-ND (**Table 2**). The similar changes in the expressions of Rho-GDI and PAK proteins were also observed in SHHR-HFDS in comparison with SD-HFDS. In addition, the protein expression of Rho GTPase-activating protein (Rho-GAP) was downregulated in the kidney of SHHRs when fed HFDS. In the category of injury of kidney, Rho-GDI protein expression decreased in the SHHR-HFDS group (**Table 3**). Since the ratio, referring to the percentage of hit proteins in the Rho-GDI signaling pathway was the highest when comparing SHHR-ND with SHHR-HFDS, we performed IPA focusing on the comparison between these groups. Rho-GDI signaling, cardiomyocyte differentiation via bone morphogenetic protein (BMP) receptors, and vascular endothelial

Fig. 1. Effects of HFDS on the body weights, the weights of kidney, visceral adipose tissue, and epididymal adipose tissue, and the serum concentrations of d-ROMs SD rats and SHHRs. SD rats and SHHRs were fed either the ND or HFDS for 6 months. The body weight (A), and the weights of the kidney (B), visceral adipose tissue (C), and epididymal adipose tissue (D) was determined. (E) The concentration of d-ROMs in serum was measured using a commercial kit. Each value represents mean ± SE (n = 7). **P < 0.01, Tukey-Kramer post hoc test.

Table 2. Highly scored proteins in the Rho-GDI signaling pathway.

Canonical pathway	Subtype	Molecule	p-value
	(1) vs (2)		
Rho-GDI signaling	SD ND vs SD HFDS	Rho-GDI (↓), PAK (↑)	1.23E-03
	SD ND vs SHHR ND	-	-
	SD HFDS vs SHHR HFDS	Rho-GDI (↓), PAK (↑)	1.23E-03
	SHHR ND vs SHHR HFDS	Rho-GDI (↓), Rho-GAP (↓), PAK (↑)	1.34E-03

Differentially expressed proteins associated with Rho-GDI signaling are listed. Upward arrows show upregulated proteins and downward arrows show downregulated proteins in the renal cytoplasm of (2) compared with that of (1). The p-value was calculated using Fisher's exact test.

growth factor (VEGF) signaling were the top 3 most significant signaling pathways that differed between the SHHR-ND and SHHR-HFDS groups (Fig. 2). These results indicate that Rho-GDI signaling would be important in the kidney under hypertensive, dyslipidemic, and hyperglycemic conditions. We next performed pathway analysis for Rho-GDI signaling. The protein expression of Rho-GDI and Rho-GAP de-

creased, and that of PAK increased in the kidney in the SHHR-HFDS group (Fig. 3). These data suggest that GTP-bound forms of Rho-GTPases would increase and activate downstream signaling in the kidneys of SHHRs fed the HFDS.

Table 3. Highly Scored Proteins in the Category of Injury of Kidney.

Category	Subtype	Molecules	<i>p</i> -value
	(1) vs (2)		
Injury of kidney	SD ND vs SD HFDS	Rho-GDI (↓), catalase (↓)	7.22E-03
	SD ND vs SHHR ND	Catalase (↓)	3.41E-03
	SD HFDS vs SHHR HFDS	Rho-GDI (↓), catalase (↓)	7.22E-03
	SHHR ND vs SHHR HFDS	Rho-GDI (↓), catalase (↓)	7.22E-03

Differentially expressed proteins included in the category of injury of kidney are listed. Downward arrows show downregulated proteins in the renal cytoplasm of (2) compared with that of (1). The *p*-value was calculated using Fisher's exact test.

Fig. 2. Highly scored canonical pathways in the kidney of SHHRs fed the ND vs HFDS by IPA.

IPA was performed using LC/MS data in the kidney cytoplasm of SHHRs fed either the ND or HFDS. The *p*-value was calculated using Fisher's exact test. The ratio shows the number of differentially expressed proteins involved in the pathway divided by the total number of proteins making up that pathway.

Gene expression of *Arhgdia*, *Dlc1*, and *Pak1* in the kidney of SD rats and SHHRs.

Proteomic analysis identified Rho-GDI, Rho-GAP, and PAK as candidate molecules affecting the kidney under hypertensive, dyslipidemic, and hyperglycemic conditions. To investigate whether the expression of these proteins is regulated at the transcriptional level, we measured the gene expression of *Arhgdia* (encoding Rho-GDI), *Dlc1* (encoding Rho-GAP), and *Pak1* (encoding PAK) using real-time PCR analysis. The expression of *Arhgdia* mRNA significantly decreased in the kidney in the SHHR-HFDS group compared with the other groups (**Fig. 4A**). The gene expression of *Dlc1* exhibited a significant decrease in both the SD-HFDS and SHHR-HFDS groups (**Fig. 4B**). These changes in mRNA expression were partially similar to those of proteins (**Table 2**), thereby indicating that the expression of

Rho-GDI and Rho-GAP proteins would be regulated at both transcriptional and translational levels. On the other hand, the gene expression of *Pak1* was parallel to its protein expression in the kidney (**Fig. 4C**, **Table 2**), suggesting that the expression of PAK would be driven at the transcriptional level in SHHRs fed the HFDS.

High expression levels of *Ngal* in the kidney of SHHRs fed the HFDS.

Because IPA pointed to the possibility that Rho-GDI signaling is activated in the kidney of SHHRs fed the HFDS, we measured the gene expression of *Ngal* in the kidney. *Ngal* gene expression significantly increased in the kidneys in the SHHR-HFDS group (**Fig. 5**).

Fig. 5. Effects of HFDS on *Ngal* gene expression in the kidney of SD rats and SHHRs.

The mRNA expression of *Ngal* was determined using quantitative PCR analysis. Data were normalized to the level of *Gapdh* mRNA expression. Each value represents mean $\pm$ SE ($n = 7$). ** $P < 0.01$, Tukey-Kramer post hoc test.

induced through Rho-GDI signaling in SHHRs fed the HFDS. Rho proteins are guanine nucleotide-binding proteins that belong to the large group of GTPases and widely involved in many biological functions¹¹. G proteins, such as Rho-GTPases, are molecular switches that cycle between an inactive GDP-bound and an active GTP-bound conformation. To turn on the switch, GTP loading to Rho-GTPases is facilitated by Rho-specific guanine nucleotide exchange factors (Rho-GEFs). Conversely, GTP hydrolysis is catalyzed by Rho-GAP to turn off the switch. Rho-GDIs dissociate inactive Rho-GTPases from the cell membrane to prevent them from misfolding and degrading, resulting in the inhibition of Rho-GTPase activities¹². On the other hand, PAK is the effector of RAC1 and CDC42 GTPases, which are Rho-GTPase family members¹³. Thus, PAK stimulates the downstream signaling of RAC1 and CDC42 GTPases. In SHHRs, the protein expression of Rho-GDI and Rho-GAP was decreased, and that of PAK was increased, in the kidney in the HFDS groups. These findings indicate that levels of the GTP-bound forms of Rho-GTPases would be increased, activating the downstream signaling in the kidney of SHHRs fed the HFDS. Previous studies revealed that the activation of Rho-GTPase leads to kidney injury^{14–16}. The loss of Rho-GDI causes progressive kidney defects that are attributable to increased RAC1 activity, ultimately leading to death in mice¹⁵. The gene expression of

Ngal, a biomarker of kidney injury¹⁷, was significantly increased in the kidney in the SHHR-HFDS group. The observation indicates that the kidney injury would be mediated through Rho-GDI signaling in SHHRs fed the HFDS. Interestingly, a recent study has demonstrated that *Arhgdia* mutations (R120X and G173V) cancel interaction with Rho-GTPases and increase GTP-bound forms of RAC1 and CDC42, but not RhoA, in patients with steroid-resistant nephrotic syndrome¹⁸. Furthermore, entire exome sequencing in two sisters with congenital nephrotic syndrome revealed that mutant Rho-GDI produced by *Arhgdia* with an in-frame deletion (D185del) is unable to bind to Rho-GTPases¹⁹. These reports support our observations that the expression of Rho-GDI decreases in the kidneys of SHHRs fed the HFDS, with concomitant kidney damage. However, we did not show the data about the direct relationship between NGAL and Rho-GDI signaling. A certification study will be needed.

It was also shown that the activation of Rho-GDI signaling is linked with hypertension, dyslipidemia, and diabetes^{20–22}. For example, RhoA kinase (ROCK) activity is enhanced in the aorta obtained from spontaneously hypertensive rats²⁰. The increased ROCK activity is significantly suppressed by the antidiabetic agent pioglitazone, accompanied by a reduction in blood pressure²⁰. Furthermore, another study suggested that simvastatin, a lipid-lowering agent, inhibits high glucose-induced Rho-GTPase/p21 signaling in glomerular mesangial cells, implying the potential for its therapeutic use in diabetic nephropathy²¹. However, no report has identified the role of Rho-GDI signaling in the kidney under hypertensive, dyslipidemic, and hyperglycemic conditions through comprehensive analysis. The present proteomic analysis emphasizes the importance of Rho-GDI signaling in the kidney of SHHRs.

In conclusion, we found that kidney injury is mediated through Rho-GDI signaling in SHHRs fed the HFDS. These findings provide insight into the molecular basis underlying the effects of hypertensive, dyslipidemic, and hyperglycemic conditions on the kidney. CKD may be prevented by ameliorating these conditions and by novel therapies targeting molecules in the Rho-GDI signaling pathway.

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