

The regulatory role of dopamine receptor D1 on PP2A via SUMO-1 modification

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Abstract. – OBJECTIVE: Renal dopamine receptor D1 played a critical role in the regulation of body blood pressure. Under hypertension, over-phosphorylation of D1 receptor impaired its function. G protein kinase 4 (GRK4) and protein phosphatase 2A (PP2A) exerted the effect to phosphorylate and de-phosphorylate D1 receptor. A current study revealed that the inhibition of GRK4 cannot normalize the phosphorylation level of D1 receptor. Meanwhile, the PP2A was activated under hypertension, indicating abnormal de-phosphorylation function of D1 receptor, the reason for which remains unknown. This study aimed to investigate the effect and mechanism of SUMO-1 modification on the regulation of dopamine receptor D1 to PP2A.

MATERIALS AND METHODS: Bioinformatics software predicted SUMO modification site in dopamine receptor D1. Cultured CHO cells were transfected with mutants of renal dopamine receptor D1. Co-immunoprecipitation and Western blot tested the interaction between over-phosphorylated D1 receptor and PP2A. Laser confocal microscopy examined their co-localization.

RESULTS: Bioinformatics predicted two SUMO modification sites K265 and K402 in dopamine receptor D1. Co-immunoprecipitation assay revealed weakened interaction between PP2A and phosphorylated D1 receptor, impeding the de-phosphorylation and normal function of D1 receptor.

CONCLUSIONS: Two SUMO modification sites existed in dopamine receptor D1, the phosphorylation of which, due to SUMO modification, can interact with PP2A, leading to the inhibition of D1 de-phosphorylation and normal function, thus providing new insights for treatment and prevention of hypertension.

Key Words

Hypertension, Dopamine D1 receptor, Protein phosphatase 2A, Phosphorylation, SUMO-1.

Introduction

Hypertension is a common disease worldwide and frequently leads to deadly complications in heart, brain or kidney, thus severely affecting public health¹. Although major advancement has

been obtained for the treatment of hypertension, the pathogenesis mechanism of which is still unclear, and overall management rate is at an unsatisfactory low level, especially in those under-developed or rural areas. Therefore, the elucidation of hypertension pathogenesis mechanism is of critical importance for the prevention and treatment of hypertension¹. Over-phosphorylation of dopamine receptor D1 is the predominant factor causing occurrence and progression of hypertension owing to dysfunction. Essential hypertension (EH) patients commonly present the symptom of sodium deposition. Kidney keeps the balance of blood pressure via regulating urine sodium reabsorption, thus drawing research interests in EH-related studies. Dopamine is an important endogenous catecholamine substance, and initially recognized as the central neurotransmitter and vascular activator. Recent reports showed that dopamine could regulate body blood pressure via the inhibition of urine sodium reabsorption². Dysfunction of dopamine/dopamine receptor is closely correlated with hypertension²⁻⁵. Dopamine receptor belonged to G protein coupled receptor of rhodopsin family, and can be classified into two groups based on pharmaceutical properties: D1 subtype (D1 and D5) and D2 subtype (D2, D3 and D4)⁶. In kidney, dopamine was mainly induced by D1 receptor with the excretion of urine sodium. The overload of sodium resulted in more than 50% excretion of sodium regulated by D1 receptor^{7,8}. Moreover, low dosage of dopamine can exert vasodilation role via stimulating D1 receptor⁹. In recent years, studies on human EH, rodent inheritance hypertensive animal model, showed that gene knockout or antagonist of D1 receptor impaired sodium/urine excretion and inhibited vasodilation effects induced by dopamine D1 receptor, under hypertensive status^{5,10-13}. Also, D1 receptor dysfunction usually emerges ahead of hypertension onset, suggesting it as a predictor before high blood pressure. To investigate possible reasons underlying D1 receptor dysfunction caused by hypertension, some studies

made comparison of the expression, distribution and phosphorylated modification of D1 receptor in proximal renal tubules (CHO) cells between EH patients and normal people, and CHO cells between normal Wistar-Kyoto (WKY) and spontaneously hypertensive rat (SHR). No significant difference of total D1 receptor expression level among those CHO cells was found, but with the elevating phosphorylated levels in D1 receptor of hypertensive CHO cells, the cell membrane distribution significantly decreased¹⁴. Phosphorylated level of D1 receptor affected its distribution on CHO cell membrane, separated D1 receptor from G protein, and eliminated the inhibitory role of D1 receptor on Na⁺-K⁺-ATPase and NHE3 sodium pumps, causing water and sodium arresting, as well as the increase of blood pressure. The phosphorylation of dopamine receptor D1 is mainly regulated by G protein related kinase 4 (GRK4) and protein phosphatase 2A (PP2A). GRK4 facilitates D1 receptor phosphorylation, and PP2A can de-phosphorylate D1 receptor. The balance between the phosphorylation and de-phosphorylation of D1 receptor determined the normal function of D1 receptor^{8,15,16}. Under hypertensive condition, hyper-active PP2A cannot efficiently de-phosphorylate those D1 receptors. Moreover, studies about PP2A found that although activity of PP2A in hypertensive CHO cells was significantly enhanced, *de facto* D1 receptor was not efficiently de-phosphorylated^{14,17,18}. Besides, the specific inhibition of GRK4 expression in SHR kidney tissues did not completely recover the phosphorylation level of D1 receptor, but only partially rescued D1 receptor function¹⁵. In this regard, this study focused to investigate the role of SUMO-1 modification on the regulation of the interplay between dopamine receptor D1 and PP2A.

Materials and Methods

Cells and reagents

CHO cells were purchased from Baili Biotech (Changchun, Jilin, China). Transfection reagent was purchased from Beyotime (Beijing, China). Anti-renal dopamine D1 receptor antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Fengshou Biotech (Shanghai, China). Anti-sense siRNA targeting renal dopamine D1 receptor and control siRNA on U6H1/

Neo plasmids were constructed and provided by Gimma (Shanghai, China). Liposome for transfection was purchased from Dingguo Bio (Beijing, China).

Cell culture

CHO cells were cultured as routine method¹⁹. DMEM (containing 20% FBS), was used for incubate cells in a 37°C chamber with 5% CO₂.

Cell transfection

24 h before transfection, cells were digested using 0.1 nM trypsin and 0.01% EDTA at 37°C for 10 min. Fresh culture medium was added for 300 g centrifugation for 5 min. Precipitated cells were re-suspended in fresh DMEM (containing 20% FBS). On the second day, when the cell density reached about 95%, liposome transfection approach was used to transfect cells with anti-sense microRNA and controlled microRNA. At 24 h after transfection, fresh culture medium was changed. 36 h later, MTT and apoptosis assay were performed.

Co-immunoprecipitation assay

Cells with successful transfection of anti-sense microRNA and controlled microRNA were collected. Western blot was used to test kidney dopamine D1 receptor and actin expression level using established methods¹⁹.

Western blot

Cells with successful transfection of anti-sense microRNA and controlled microRNA were collected. Western blot was used to test kidney dopamine D1 receptor and osteopontin expression level using established methods¹⁹. In brief, total cell protein suspension was prepared following manual instruction of test kit. Protein content was measured by BCA kit¹⁸. Cell lysate was extracted and quantified in a micro plate reader. Protein was separated and boiled for 10 min for Western blot using 12% separation gel (60 V for 30 min, followed by 120 V for 90 min). The protein was then transferred to the membrane under 90 mA for 180 min. The membrane was blocked in 5% defatted milk powder for 60 min incubation at room temperature. Primary antibodies (1:1000) against dopamine D1 receptor and housekeeping protein actin were added for 4°C overnight incubation. TBST was used to wash excess primary antibody, followed by the addition of secondary antibody (anti-mouse IgG at 1:2500). After 3 h incubation under

37°C, TBST was used to wash the membrane, which was then stained in horseradish peroxidase (HRP) and was developed in ECL chromogenic substrate. A gel imaging system was used to quantify gray values. Images were taken by the build-in system (Shanpu, Shanghai, China) to analyze protein expression level²⁰.

Confocal microscopy

CHO cells were cultured by routine method. Cells were fixed in 4% paraformaldehyde for 20 min, followed by three times of PBS washing. 0.1% Triton-100 was used for the treatment of 10 min. After three times of PBS rinsing, 5% BSA was added for blocking overnight. After three times of PBS washing, mouse anti-human secondary antibody (1:200) was added for 1 h incubation, followed by three times of washing (5 min). Fluorescent spot was visualized under microscope.

Statistical Analysis

Each experiment was performed in triplicates. Results were analyzed by SPSS 13.0 (SPSS Inc., Chicago, IL, USA). All experimental data were presented as mean±standard deviation (SD). Comparison among groups was performed using one-way analysis of variance (ANOVA). LSD and S-N-K tests were provided as equal variances and were assumed, while Tamhane's T2 test was used as equal variances, and were not assumed. A statistical significance was defined when $p < 0.05$.

Results

Bioinformatics software prediction

Two online SUMO site prediction software, SUMOplotTM Analysis (<http://www.abgent.com/sumoplot.html>) and SUMOsp 2.0 (<http://sumosp.biocuckoo.org/online.php>) both predicted common SUMO modification sites (K265 and K402) in dopamine D1 receptor on human and rats. As shown in Figure 1, both human and rat dopamine D1 receptors share common SUMO modification sites (K265 and K402, Figure 1). These sites were proximal to previously reported major phosphorylated sites in D1 receptor, including S263, T268, S397 and S398. Therefore, there is a possible interaction between phosphorylation and SUMO modification sites in D1 receptor. Bioinformatics prediction revealed two SUMO modification sites- K265 and K402- in dopamine D1 receptor.

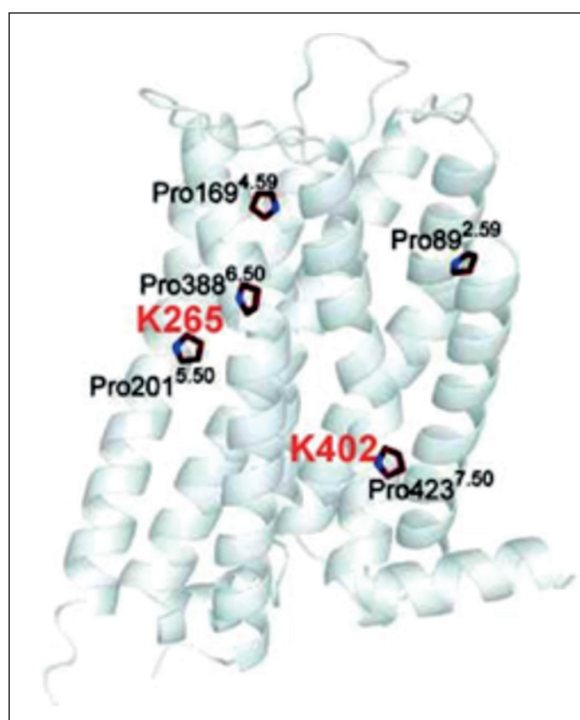


Figure 1. Prediction by bioinformatics software. Both human and rat dopamine D1 receptors share common SUMO modification sites (K265 and K402).

Co-immunoprecipitation results

Based on the bioinformatics prediction results, CHO cells were then transfected with plasmids expressing dopamine D1 receptor, co-expressing SUMO-1 and dopamine D1 receptor, or co-expressing SUMO-1 and D1 receptor mutants. Both Western blot and co-immunoprecipitation approaches were used to compare the modification of D1 receptor by SUMO-1 to determine the functioning sites. As shown in Figure 2, co-immunoprecipitation results indicated differential phosphorylation level on dopamine D1 receptor in CHO cells of SHR and WKY rats after transfection. The phosphorylation level in D1 receptor of SHR cells was found higher than that in WKY cells. The weakened interaction between over-phosphorylated D1 receptor and PP2A was observed, along with enhanced SUMO-1 modification potency.

Reverse co-immunoprecipitation assay

As shown in Figure 3, the interaction between dopamine D1 receptor and PP2A was further investigated by co-immunoprecipitation analysis. Results indicated that compared to WKY, SHR cells had weakened interaction between D1 re-

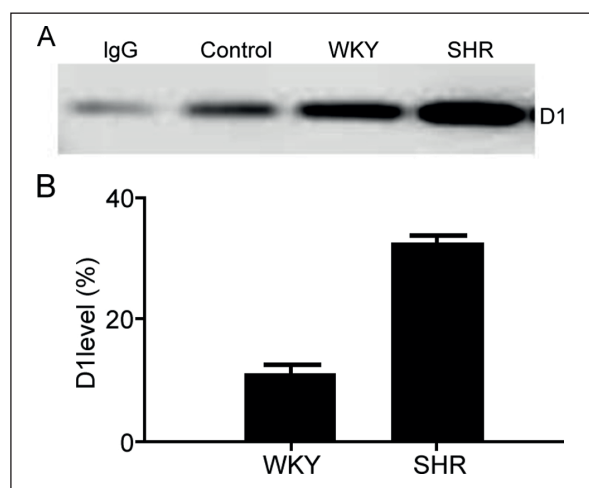


Figure 2. Dopamine D1 receptor phosphorylation level in CHO cells from WKY and SHR animals. *, $p < 0.05$ comparing between SHR and WKY (N=6). CHO cells from SHR animals showed higher D1 receptor phosphorylated level than WKY.

ceptor and PP2A. The weakened self SUMO-1 modification and over-phosphorylation in D1 receptor potentiated interaction with PP2A.

Co-immunoprecipitation assay for GRK4

The phosphorylation level of D1 receptor was up-regulated by co-transfection of dopamine D1 receptor and GRK4, whilst the level was suppressed by transfection of GRK4-siRNA. As shown in Figure 4, the phosphorylation level of dopamine D1 receptor in CHO was elevated after the transfection of GRK4, along with weakened interaction between D1 receptor and PP2A. Under hypertensive status, over-phosphorylation of D1 receptor induced SUMO modification and impeded the interaction between PP2A and over-phosphorylated D1 receptor.

Co-expression and co-localization of dopamine D-1 receptor and SUMO-1 in SHR and WKY cell lines

As shown in Figure 5, immune fluorescent staining coupled with laser confocal microscopy revealed the co-expression and co-localization between dopamine D1 receptor and SUMO-1 in SHR and WKY cell lines. Co-transfection of dopamine D1 receptor and GRK4 in CHO cell line up-regulated the phosphorylated level of D1 receptor. The over-phosphorylated D1 receptor weakened the interaction between D1 and PP2A, along with enhancing self-modification of SUMO-1.

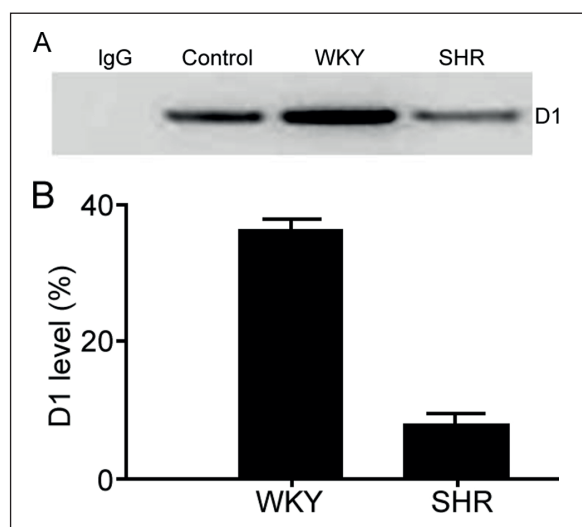


Figure 3. Interaction between dopamine D1 receptor and PP2A in CHO cells from WKY and SHR. *, $p < 0.05$ comparing between SHR and WKY (N=6). Comparing to WKY, SHR CHO cells showed weakened interaction between dopamine D1 receptor and PP2A.

Modification of D1 receptor by SUMO-1 in CHO cells after single transfection of D1 receptor, co-transfection of D1 receptor plus SUMO-1 and co-transfection of D1 receptor, GRK4 plus SUMO-1

As shown in Figure 6, co-immunoprecipitation described SUMO-1 modification in D1 receptor of CHO cells with transfection by D1 receptor alone, D1 receptor + SUMO-1, or D1 receptor GRK4 + SUMO-1. These results further showed weakened interaction between over-phosphorylated D1 receptor and PP2A, with increasing SUMO-1 modification.

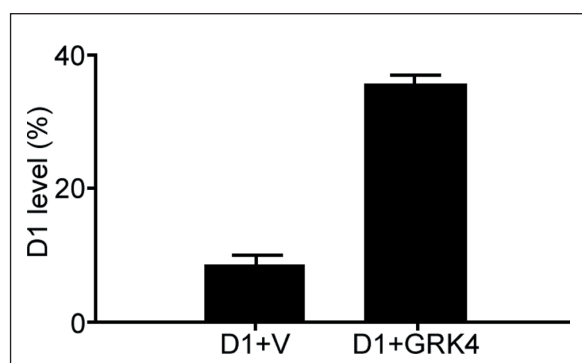
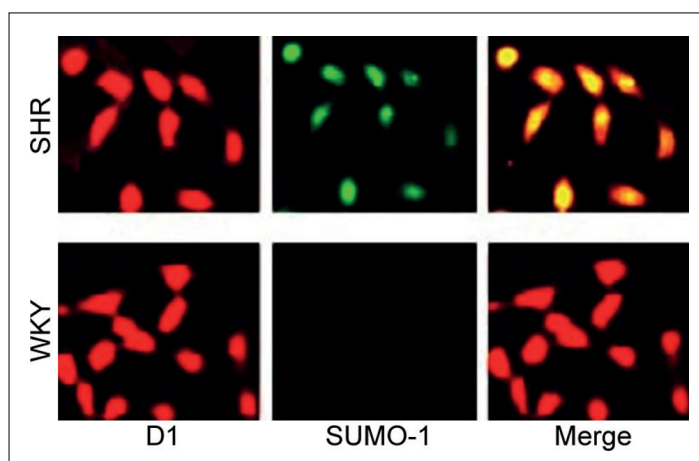


Figure 4. Weakened interaction between dopamine D1 receptor and PP2A after transfecting CHO cells with GRK4 to elevate phosphorylated level of dopamine D1. *, $p < 0.05$ compared to those cells only transfected with dopamine D1 receptor (N=6).

Figure 5. Immune fluorescent staining and laser confocal microscopy revealed co-expression and co-localization in SHR and WKY cell lines.

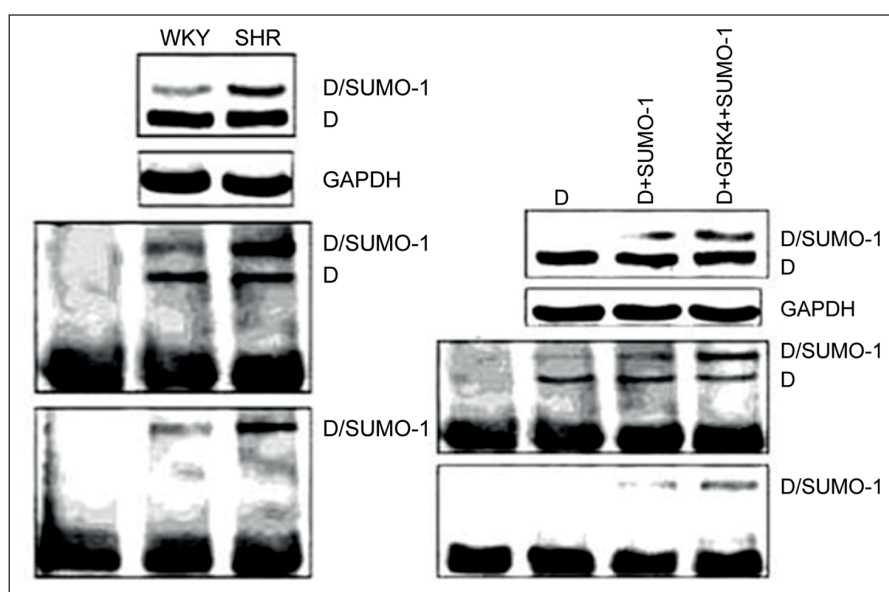


Discussion

Currently, the incidence of hypertension is significantly increasing both in China and in the world. In China, the overall incidence of hypertension is about 18.8%, as more than 200 million people have been suffering from it¹. Study on SUMO modification found that it could regulate interaction between proteins²¹. SUMO is characterized as important post-translational modification of proteins. During SUMO, carboxyl group in glycine residue binds to ϵ -amino group of lysine residue, thus affecting the stability of protein substrate, sub-cellular localization, transcriptional activity and interaction with other proteins, for the purpose of the modulation of protein function^{21,22}. A

total of four SUMO genes have been discovered in mammalian cells, including SUMO-1, -2, -3 and -4^{21,22}. SUMO-1, -2 and -3 are highly expressed in most tissues, whilst SUMO-4 is mainly expressed in kidney, lymph node and spleen tissues^{19,20}. SUMO-1 primarily exists in the form of binding to substrate protein, whilst SUMO-2/3 appears in a free form. Whether SUMO-4 has biological functions is still inconclusive^{21,22}. A current study has confirmed that phosphorylation of substrate protein can modify the interaction between proteins via mediating self SUMO modification. In this study, we compared the interaction between PP2A and D1 receptor in CHO cells from WKY and SHR animals. For the first time we found significantly enhancing the phosphorylation level of D1 receptor

Figure 6. Enhanced modification of D1 receptor by SUMO-1 in CHO cells after single transfection of D1 receptor, co-transfection of D1 receptor plus SUMO-1 or co-transfection of D1 receptor, GRK4 plus SUMO-1 (N=6).



in SHR CHO cells, whilst the interaction between PP2A and D1 receptor was significantly weakened. Moreover, we mimicked the over-phosphorylation of D1 receptor by co-transfecting GRK4 and D1 receptor, and also found weakened interaction between phosphorylated D1 receptor and PP2A. Under hypertension status, the dephosphorylation of D1 receptor was inhibited, due to weakened interaction between PP2A and D1 receptor. Kidney dopamine D1 receptor is the critical protein regulating cell apoptosis¹¹⁻¹⁵. Whether kidney dopamine D1 receptor participates in hypertension is still unclear. Therefore, this study utilized CHO cell model, on which anti-sense microRNA targeting kidney dopamine D1 receptor was used in transfection to investigate the regulatory role of kidney dopamine D1 receptor on CHO cells. Bioinformatics software predicted the existence of two SUMO modification sites – K265 and K402 – in dopamine D1 receptor. In our study, the SUMO modification contributed to the phosphorylation of dopamine D1 receptor and its interaction with PP2A, demonstrating the function of SUMO in physiological and pathological processes as previously described²³. However, the limitation in our work existed that the level of dopamine D1 receptor in kidney should be evaluated in animals model at different developmental stages, and extensive samples from hypertensive patient ought to be collected to further validate results of animal experiments, in order to further validated the correlation between kidney dopamine D1 receptor level and hypertension.

Conclusions

This paper showed two SUMO modification sites in dopamine D1 receptor. SUMO modification affected the phosphorylation of dopamine D1 receptor and the interplay with PP2A, leading to the inhibition of de-phosphorylation and dysfunction of D1 receptor. These results provide new insights for prevention and treatment of hypertension.

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Conflict of Interests:

All authors declare no conflict of interest.

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