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Research report

Salt-induced phosphoproteomic changes in the hypothalamic paraventricular nucleus in rats with chronic renal failure

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ABSTRACT

Hypothalamic paraventricular nucleus (PVN) is a cardiovascular regulating center within the brain, which plays a critical role in high salt-induced progression of chronic renal failure (CRF). However, the phosphoproteomic changes in the PVN caused by CRF remain unclear. This study aimed to perform large-scale phosphoproteomic analysis of PVN induced by CRF and high salt intake. In this study, eight weeks post 5/6 nephrectomy (CRF model) or sham operation, Sprague–Dawley rats were fed a high-salt (4%) or normal-salt (0.4%) diet for 3 weeks. TiO_2 enrichment, iTRAQ labeling, and liquid chromatography tandem mass spectrometry were applied for phosphoproteomic profiling of PVN. A total of 3723 unique phosphopeptides corresponding to 1530 phosphoproteins were identified. Compared with sham group, 133 upregulated and 141 downregulated phosphopeptides were identified in CRF group during normal-salt feeding. However, with a high-salt diet, 160 phosphopeptides were upregulated and 142 downregulated in the CRF group. Gene Ontology analysis revealed that these phosphoproteins were involved in binding, catalytic, transporter, and other molecular functions. Search Tool for the Retrieval of Interacting Genes protein–protein analysis showed direct or indirect functional links among 25 differentially expressed phosphoproteins in CRF rats compared with sham group. However, 24 differentially phosphorylated proteins induced by high salt intake were functionally linked in CRF animals. The altered phosphorylation levels of dynamin 1, TPPP and Erk1/2 were validated. Phosphoproteomic changes of PVN triggered by CRF and high salt-load have been investigated. It will provide new insight into pathogenetic mechanisms of development of chronic kidney disease and salt sensitivity.

1. Introduction

Chronic kidney disease (CKD) is a global public health problem. Many countries, such as USA, China, and Australia, have a high prevalence of CKD (Li et al., 2014; Zhang et al., 2012; Mallett et al., 2014). Moreover, CKD is a major contributor to global noncommunicable diseases. Its occurrence and development involve pathological changes in multiple organs, including the cardiovascular system and the brain (Couser et al., 2011). Studies have shown that the PVN of the hypothalamus is a principal cardiovascular site. It receives afferent inputs from other regions and translates them into changes in single specific outputs, such as neuroendocrine and autonomic, to regulate the functions of downstream organs (Ferguson et al., 2008). The sympathetic outflow and activation of PVN neurons increase in rats with chronic heart failure, and the activation of PVN neurons can promote sympathetic discharge (Xu et al., 2012). CKD is accompanied by increased sympathetic activity, and renal denervation can improve renal pathological changes (Hering et al., 2013). The PVN can directly regulate the renal afferent sympathetic nerve activity through its anterior sympathetic neurons (Akine et al., 2003). It can also regulate the action of glucocorticoids and antidiuretic hormone, indirectly affecting the cardiovascular function (Benarroch, 2005). Our recent study demonstrated that high salt induced overactivation of renin–angiotensin system in the PVN of CRF rats. It activated the sympathetic nervous system and promoted oxidative stress, fibrosis, and progression of CKD (Cao et al., 2015). Large

Abbreviations: PVN, hypothalamic paraventricular nucleus; CRF, chronic renal failure; iTRAQ, isobaric tags for relative and absolute quantification; CKD, chronic kidney disease; MS, mass spectrometry; (LC)-MS/MS, liquid chromatography tandem mass spectrometry; SBP, systolic blood pressure; 5/6 Nx, 5/6 nephrectomy; NS, sham operation + normal salt diet; NC, 5/6 Nx + normal salt diet; HC, 5/6 Nx + high salt diet; AD, Alzheimer's disease; KEGG, Kyoto Encyclopedia of Genes and Genomes.

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numbers of uremic toxins produced in CKD contribute to neurohumoral dysfunction by regulating intracerebral cellular and molecular pathways and increasing the sensitivity of the central nervous system to extracerebral signals including angiotensin II and the afferent renal nerves in the PVN (Underwood et al., 2016).

Patients with CKD generally have elevated blood pressure (Ihm, 2015). The increased dietary salt intake is an important risk factor for high blood pressure and cardiovascular disease (Strazzullo et al., 2009). It has also been demonstrated that patients with CKD are more likely to be salt sensitive (Weir and Fink, 2005), and salt restriction can improve the renal impairment (Swift et al., 2005). Increased salt consumption in patients with CKD has been demonstrated to result in higher filtration fraction, greater intraglomerular pressure, and proteinuria (Weir et al., 1995). Moreover, studies have indicated that the development of interstitial fibrosis and the deterioration of renal function is attributed to the salt-induced increases in reactive oxygen species (Wilcox, 2002). The progression of CKD caused by high salt intake aggravated the lesions of PVN, resulting in enhanced inflammation, increased reactive oxygen species, increased renin–angiotensin system activation of local regions, and hypersecretion of arginine vasopressin(Cao et al., 2015). These events contribute to the deterioration of renal function by stimulating sympathetic activation and elevating blood pressure.

Protein phosphorylation is a post-translational modification important for cellular regulation and signal transduction. It is implicated in several cellular processes from proliferation and differentiation to apoptosis. Numerous studies have shown that protein phosphorylation plays a crucial role in the functional regulation of PVN. Previous studies have found that the PVN plays a key role in physiological homeostatic control. The protein kinases and signaling pathways associated with protein phosphorylation, include protein kinase C, calcium/calmodulin-dependent protein kinase (CaMK) II, protein kinase A (PKA), mitogen-activated protein kinases (MAPKs) p44/42, p38, c-Jun N-terminal kinase (JNK), phosphatidylinositol-3-kinase (PI3K), and serine/threonine kinase (Akt). These are also involved in sympathetic overdrive, arginine vasopressin secretion, and angiotensin II–induced upregulation of angiotensin II type 1 receptor (AT1R), and all have a corresponding change in the expression or activity (Sun et al., 2016; Yoshida, 2008; Wei et al., 2008). This suggests that attention needs to be paid not only to the changes in protein expression before and after the disease but also to the phosphorylation-based signaling when analyzing the underlying mechanisms of PVN lesions and dysfunction in CKD and high salt intake. However, the levels of protein phosphorylation in the PVN caused by chronic renal failure (CRF) are still unexplored.

The present study aimed to perform a large-scale phosphoproteomic analysis of PVN in rats with CRF and a salt-load rat model of CRF. The isobaric tags for relative and absolute quantification (iTRAQ) technology combined with liquid chromatography–tandem mass spectrometry (LC-MS/MS) and TiO_2 enrichment were used to investigate the changes (Unwin et al., 2010; Larsen et al., 2005). A large number of phosphopeptides corresponding to 1530 phosphoproteins, involved in different signaling pathways or biological processes in the PVN caused by CRF or high salt intake, were identified based on previous studies. These phosphoproteins may serve as targets for the mechanisms of CKD in the central nervous system.

2. Results

2.1. Physiological parameters

The body weight, systolic blood pressure (SBP), and serum creatinine in rats who underwent 5/6 nephrectomy and control rats were examined to ensure the successful establishment of the CRF model. Table 1 shows the changes in the physiological parameters among different groups. The differences were statistically significant. Rats with CRF showed higher SBP and serum creatinine compared with controls. After 3-weeks, salt loading resulted in significantly greater SBP levels in the rats, who underwent 5/6 nephrectomy, with high salt intake compared with the levels in those with normal salt intake. Moreover, high salt intake induced a more pronounced increase in kidney weight/body weight ratio.

Table 1
Changes in biochemical and metabolic parameters.

	Sham +normal salt	5/6Nx +normal salt	5/6Nx +high salt
Body wt (g)			
0 week	463 ± 10.1	406 ± 6.6[a]	412 ± 14.9[a]
3 week	505 ± 17.3	425 ± 7.4[a]	419 ± 29.9[a]
Kidney wt/body wt (mg/g)			
	3.3 ± 0.18	4.4 ± 0.16[a]	5.7 ± 0.41[a,b]
Serum creatinine (μmol/ L)			
0 week	32 ± 1.1	77 ± 8.5[a]	78 ± 6.7[a]
3 week	35 ± 1.2	112 ± 16.7[a]	135 ± 21.4[a]
SBP (mmHg)			
0 week	120.9 ± 1.9	144.2 ± 3.0[a]	145.4 ± 2.7[a]
3 week	119.1 ± 1.6	145.5 ± 4.0[a]	173.2 ± 3.7[a,b]

All data are expressed as mean ± SEM ($n = 8$ in each group).
[a] $p < 0.05$ vs sham rats + normal salt.
[b] $p < 0.05$ vs CRF rats + normal salt; 0 week, 8 weeks after the last surgery; 3 week, 11 weeks after the last operation; kidney wt/body wt, kidney weight/ body weight; SBP, systolic blood pressure.

2.2. Identification of phosphorylated proteins and sites

iTRAQ combined with LC-MS/MS was used to identify and quantify the phosphopeptides. Also, TiO_2 was used to enrich the phosphopeptides. Supplementary Table S1 shows the identified phosphopeptides in detail. In this study, 3723 unique phosphopeptides corresponding to 1530 phosphoproteins were identified (Supplementary Tables S1 and S2). The post-translational modification score was used as a reference point to more accurately assign phosphorylation sites identified by the tandem mass spectra as described previously. Among the identified phosphorylation sites (Fig. 1a and b), 4548 phosphorylated sites were localized with high confidence, in which the number of phosphoserine, phosphothreonine, and phosphotyrosine residues was 4019, 513, and 16, respectively. The percentages of the identified phosphopeptides with singly, doubly, triply, and more highly phosphorylated sites were 67.77%, 26.82%, 4.22%, and 1.19%, respectively.

The frequency distribution of phosphopeptides at NC/NS or HC/NC ratio was drawn as a histogram (Fig. 1c and d). The pictures showed that the phosphorylated peptides were mainly distributed at NC/NS and HC/NC ratio of approximately 1. The quantitative ratio distribution of differentially phosphorylated peptides (Supplementary Tables S3) indicated that 1.5-fold upregulaton or downregulation in different groups was 126 (NC vs NS group) and 216 (HC vs NC group) (Fig. 1e and f).

2.3. Differentially phosphorylated peptides in response to CRF and salt load

The hierarchicallyclustered heatmaps of the differentially phosphorylated peptides for NC/NS and HC/NC comparison groups showed that the differentially phosphorylated peptides were coordinately regulated by CRF and high salt intake (shown in Supplementary Figs. S1 and S2). These identified differentially phosphorylated proteins, were annotated with the gene ontology (GO) format using the Protein ANalysis THrough Evolutionary Rela-