Chronic renal failure induces cell death in rat hippocampal CA1 via upregulation of αCaMKII/NR2A synaptic complex and phosphorylated GluR1-containing AMPA receptor cascades

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Abstract

Background: N-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methylisoxazole-4-propinoic acid (AMPA) receptors bound to postsynaptic density-95 (PSD-95) and α isoform of calcium/calmodulin-dependent protein kinase II (αCaMKII) is fundamentally involved in the regulation of working memory. The aim of present study was to investigate the alterations of NMDA and AMPA receptors responsible for hippocampal synaptic dysfunction and selective neuronal cell death after chronic renal failure (CRF) which may be associated with impairment of working memory.

Methods: Altered interactions between NMDA and AMPA receptors and PSD-95 and αCaMKII were analyzed in the cornu ammonis (CA) 1 and CA3/dentate gyrus (DG) subfields of the uremic rat hippocampi using the immunoblotting and immunoprecipitation methods.

Results: Uremia induced by CRF produced necrotic cell death and decreased neuronal nucleoli protein levels in the hippocampal CA1 subfield, but not in the CA3/DG subfields. The CA1 subfields of CRF rats exhibited significant decreases and increases, respectively, in the expressions of PSD-95/NR2B and αCaMKII/NR2A synaptic complex. Moreover, increased phosphorylation of glutamate receptor type 1 (GluR1) AMPA receptor at ser831 was observed in the CA1 subfield after CRF.

Conclusion: These hippocampal CA1 neuronal vulnerability may be responsible for memory dysfunction after CRF as mediated by an increase in NR2A-containing NMDA receptors bound to αCaMKII and subsequent activation of GluR1-containing AMPA receptors caused by the phosphorylation of GluR1 at ser831.

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Introduction

In science, cognition is the mental processing that includes the attention of working memory, comprehending and producing language, calculating, reasoning, problem solving, and decision making [1]. The hippocampus belongs to the limbic system and plays important roles in the consolidation of spatial working memory. Moreover, hippocampal volume can be used to detect cognitive dysfunction and identify elderly people at risk of Alzheimer’s disease [2]. However, the selective vulnerability of hippocampal neurons in chronic renal failure (CRF) remains speculative even though the hippocampus seems particularly susceptible to a variety of uremic toxins and metabolites [3]. Clinical studies have demonstrated a high risk for dementia and cognitive impairment in patients with chronic kidney disease (CKD) and those undergoing hemodialysis [4,5]. Even in patients with neurologically asymptomatic chronic renal disease, impaired cognitive processing can be disclosed by event-related potentials [4]. In animal studies, evidence indicates that uremia impairs synaptic transmission in the rat hippocampus, and that this results in increased glutamate release presynaptically, which in turn, impairs spontaneous synaptic activity [6]. These observations suggest that marked elevations in uremic toxin levels functionally perturb synaptic function of the hippocampus and leads to neuronal cell death and subsequent cognitive dysfunction.

N-methyl-D-aspartate (NMDA) receptors mediate long-term potentiation which is a candidate mechanism for memory [7], and play critical roles in synaptic dysfunction and neuronal cell death [8]. In the hippocampus, the NMDA receptors are composed of NR1 and NR2 subunits (NR2A, NR2B), which cluster with scaffolding proteins, such as, post-synaptic density-95 (PSD-95) and α isoform of calcium/calmodulin-dependent protein kinase II (αCaMKII) [9]. Previous studies have reported that the dynamic but independent regulations of the NR2A and NR2B subunits by αCaMKII and PSD-95, respectively, are modulated by competitive or antagonistic interactions between these two scaffolding proteins [10]. Furthermore, it has been shown that different levels of NR2A and NR2B at synapses differentially regulate the surface expression of glutamate receptor type 1 (GluR1)-containing 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid (AMPA) receptor via the Ras-activated extracellular signal-regulated kinase (ERK) pathway, which is essential for the modification of synaptic strength [11].

AMPA receptor is an ionotropic glutamate receptor that functions in fast synaptic transmission and plasticity [12]. At the excitatory synapses of central neurons, AMPA receptors are organized into multiprotein signaling complexes within the postsynaptic density [12]. In a previous study, it was demonstrated that protein kinase G regulates AMPA receptor trafficking by modulating GluR1-ser845 phosphorylation in hippocampal slices [13], and in another, it was shown that the αCaMKII–mediated Ser831 phosphorylation of GluR1 is associated with AMPA receptor expression and potentiation in the postsynaptic membrane [14]. However, diminished αCaMKII levels result in the detachment of GluR1 subunit-containing AMPA receptors from cell surfaces and in the attenuation of AMPA receptor currents [15].

Accordingly, alterations in the levels of postsynaptic proteins in the hippocampus can significantly affect cognitive impairment through the synaptic dysfunction and subsequent neuronal vulnerability, and thus could contribute importantly to uremic encephalopathy in CRF. In this study, changes in the protein expressions of postsynaptic proteins and their interactions in the hippocampi of CRF rats were examined.

Methods

Experimental animals

Studies were performed using adult male Sprague–Dawley rats weighing 290 ± 8 g. Animals were maintained on a standard rodent diet with free access to water. The experimental procedures used were reviewed and approved by the Animal Care and Use Committee of Dongguk University, Gyeongju, Korea. Animal care and use were also in accord with the guidelines issued by the National Institute of Health (Bethesda, MD, USA).

Induction of CRF by surgical reduction

Experimental CRF was induced by excising approximately two-thirds of the left kidney and performing right total nephrectomy [16]. The protocols used in this study are depicted in Fig. 1. Rats were anesthetized with 3% isoflurane in a mixture of oxygen/nitrous oxide (30:70) and maintained on 1.5% isoflurane. During surgery, a heating pad was used to maintain a rectal temperature of 37–38 °C. The left kidney was exposed via a left flank incision and gently dissected free from the adrenal gland. Approximately two-thirds of the left kidney, including the upper and lower poles, was then excised. One week after initial surgery, again under isoflurane anesthesia, right kidneys were excised (after release from the adrenal gland) and removed through a right flank incision. Some rats were subjected to an identical sham operation, but kidneys were left intact. Sham-operated rats and 5/6 nephrectomized CRF rats were monitored for 7 weeks after surgery for establishing the long-term effects of chronic kidney disease on the brain. Cornu ammonis (CA) 1 and CA3/ dentate gyrus (DG) tissue sections of hippocampus were separated under the surgical microscopy and then rapidly frozen at −80 °C until required for protein assays.

Figure 1. Diagram of the excision remnant kidney model. Experimental chronic renal failure (CRF) was induced by two-stage excision of about two-thirds of the left kidney and right total nephrectomy. The left kidney was exposed using a left flank incision and gently dissected free from the adrenal gland; approximately two-thirds of the left kidney, including the upper and lower pole was excised. One week later, the right kidney was removed via a right flank incision after dissecting it free from the adrenal gland. Rats were maintained in metabolic cages on the days marked with an asterisk. As controls, some rats were subjected to a sham operation identical to that described above but without kidney tissue removal.
Plasma and urine analyses

Twenty-four-hour urine samples were collected in metabolic cages on the last day of the experiment. Blood samples were taken from abdominal aortas under isoflurane-anesthesia and heparinized (10 IU/mL of blood). Plasma was immediately separated by centrifugation (2,000 × g for 3 minutes at 4°C). Urine and plasma samples were stored in a freezer (−80°C) until required for chemical analyses. Hematocrit, plasma creatinine, and urinary creatinine clearance were measured using conventional methods.

Cresyl violet staining

Sham-operated control rats (n = 3) and 5/6 nephrectomized CRF rats (n = 3) were anesthetized and the brain was fixed by a transcardiac perfusion of 4% paraformaldehyde in phosphate buffered saline (PBS) after removal of blood from the cerebral vasculature by perfusion of physiologic saline. The brain was removed and postfixed in the same fixative for 12 hours. Perfusion fixed brain tissues were paraffin-embedded and serial coronal sections (5 μm thickness) were obtained at the level of dorsal third ventricle (bregma−4.16 mm). Paraffin wax was removed by xylene overnight at room temperature and the sections were rehydrated with ethanol (99%, 96%, and 70%). After washing in distilled water, the sections were stained with cresyl violet for 30 minutes at room temperature. The sections were then treated successively with ethanol (99%, 96%, and 70%) and differentiator (glacial acetic acid and 95% ethanol).

Hippocampal homogenate preparation

For protein extraction, each hippocampal CA1 and CA3/DG subfield tissue sections were homogenized in ice-cold homogenizing buffer [50mM HEPES, 50mM NaF, 20mM sodium pyrophosphate (NaPi), 1mM EDTA, 8.5mM leupeptin, and 1mM phenylmethylsulfonyl fluoride (PMSF); pH 7.4; n = 16; sham operated control rats (n=8) and CRF rats (n=8)]. Protein contents were measured using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

Semiquantitative immunoblotting

The levels of proteins in hippocampal samples were determined by immunoblotting under conditions of protein linearity. After sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), proteins were transferred to nitrocellulose membranes in a buffer solution containing 50mM tris-base, 380mM glycine, and 20% methanol. Membranes were then blocked with 5% milk in PBS-T (80mM Na2HPO4, 20mM NaH2PO4, 100mM NaCl, 0.1% Tween 20; pH 7.5) for 1 hour and incubated overnight at 4°C with mouse antineuronal nuclei (NeuN; 1:1,000; Chemicon, Temecula, CA, USA), rabbit antiPSD-95 (1:1,000), mouse anti-CaMKII (1:2,000), or mouse anti-p-Ser831 and 845 GluR1 (1:1,000; Santa Cruz Biotechnology, Texas, USA). Sites of antibody–antigen reaction were visualized using horseradish peroxidase (HRP)-conjugated secondary antibodies (P447 or P448, diluted 1:3,000; DAKO, Glostrup, Denmark) and an enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech, Little Chalfont, UK), and then exposed to photographic film (Hyperfilm ECL, RPN3103K, Amersham Pharmacia Biotech). Immunoblot signals developed using the ECL system were quantified using Scion Image software (version 1.59; Bethesda, Maryland, USA).

Immunoprecipitation

Hippocampal CA1 homogenates (600 μg) were diluted fourfold with 50mM HEPES buffer (10% glycerol, 150mM NaCl, 0.5% NP-40, 10mM Tris–HCl, 1.0mM EDTA, 1.0mM PMSF 1.0% Triton Maryand, USA).

![Figure 2. Neuronal cell death in the hippocampal CA1 subfield after CRF was determined by cresyl violet staining and immunoblotting.](#) Different subpopulations of hippocampal cells were chosen to characterize the cellular changes under CRF (X4). Rectangle indicates the necrotic subfield in the hippocampus. (B, D, F) There were no necrotic cells in the sham-operated rat hippocampus. (C) Cells in the CA1 subfield undergo typical necrotic cell death. (E, G) In contrast, the cells in the CA3/dentate gyrus region were more resistant to necrotic cell death (X20). Control n=3, CRF n=3. (H) Immunoblotting for neuronal nuclei (NeuN) protein revealed a major strong band at 32 kDa in sham-operated controls. α-actin was used as an internal control to confirm equal protein loading. (I) Immunoblotting analyses showed that the expressions of NeuN in the CA1 subfield were significantly decreased after CRF. In contrast, levels of NeuN in the CA3 and DG subfields were unchanged after CRF. Results are means ± SEMs. Control n=8, CRF n=8. *P < 0.01. CA1, cornu ammonis 1; CA3, cornu ammonis 3; CRF, chronic renal failure; DG, dentate gyrus.
X-100, and 1.0% sodium deoxycholate; pH 7.4), and then affinity-purified rabbit anti-PSD-95 (Upstate Biotechnology, Lake Placid NY, USA) and αCaMKII antibodies (Affinity Bioreagents, Golden Colorado, USA; both 1:50) were added. After incubating mixtures for 4 hours at 4°C, protein A-agarose slurry (1:1) was added and incubated for a further 2 hours. Samples were then centrifuged at 100,000 × g for 2 minutes and the supernatants so obtained were applied to SDS-gels and electrophoresed onto nitrocellulose membrane for immunoblotting with anti- NR2A antibody or anti-NR2B antibody (1:1000; Upstate Biotechnology, Lake Placid NY, USA).

Data analysis

Values are presented as mean ± standard error of mean (SEM). Groups were compared using one-way analysis of variance (ANOVA) followed by Bonferroni’s post hoc test. The statistical differences were considered significant if the P values less than 0.05 or 0.01.

Results

Establishment of CRF induced by 5/6 nephrectomy

Seven weeks after 5/6 nephrectomy, CRF rats lost ~9% of body weight as compared with the sham-operated controls. Diminished renal function was demonstrated by significant increases in plasma creatinine levels (8.75 ± 0.47 in CRF vs. 1.13 ± 0.55 mg/dl in sham-operated controls; P < 0.05) and urine outputs (61.4 ± 10.9 in CRF vs. 45.9 ± 3.1 ml/kg/d in sham-operated controls; P < 0.05). Urinary creatinine clearance was also significantly decreased (4.45 ± 0.32 in CRF vs. 7.32 ± 0.83 ml/kg/d in sham-operated controls; P < 0.05). In addition, CRF rats had lower hematocrit levels (37.4 ± 0.6 in CRF vs. 42.3 ± 0.6 in sham-operated controls; P < 0.05).

Neuronal cell death in the CA1 subfield of uremic hippocampi

Cresyl violet staining demonstrated that cells in the medial CA1 subfield of hippocampus (Fig. 2A, rectangle) underwent typical necrotic cell changes, such as dark in color, irregular shape, cork-screw processes, and expansion of perineuronal fields. By contrast, cells in the CA3 subfield and DG region were more resistant and less necrotic cell death was observed (Fig. 2E and G). Semiquantitative immunoblotting for neuronal nuclei (NeuN) protein revealed a major band at 32 kDa in sham-operated controls (α-actin was used as internal loading control; Fig. 2H). Immunoblotting demonstrated that the expressions of NeuN in CA1 subfield homogenates were significantly reduced by CRF (to 72 ± 6.3% vs. sham-operated controls, n = 8, P < 0.01). By contrast, levels of NeuN in CA3 and DG subfields were not changed by CRF (Fig. 2I).

Figure 3. Normalized expressions of postsynaptic density-95 (PSD-95) and α postsynaptic density-95 (PSD-95) in CA3/DG subfields after CRF. (A) Immunoblot with anti-PSD-95 and αCaMKII antibodies revealed 95 and 50 kDa bands, respectively, in sham-operated and CRF rats. (B) In CA1 subfields, densitometric analyses revealed that CRF significantly decreased PSD-95 expression but significantly increased αCaMKII expression. The expressions of PSD-95 and αCaMKII after CRF were unchanged in the CA3/DG subfields. Results are means ± SEMs. Control n = 8, CRF n = 8. *P < 0.01. CA1, cornu ammonis 1; CA3, cornu ammonis 3; CRF, chronic renal failure; DG, dentate gyrus; SEM, standard error of mean.

Figure 4. CRF induced changes in bindings between PSD-95 or αCaMKII and NMDA receptor subunits (NR2A and NR2B) in the rat hippocampal CA1 subfield. (A) Homogenate samples from sham-operated and CRF rats were immunoprecipitated with anti-PSD-95 and anti-αCaMKII antibodies, respectively. Immunoprecipitates were analyzed by immunoblotting with anti-NR2A or anti-NR2B antibodies. (B) Quantitative analysis of immunoblots showed the amount of NR2B that co-precipitated with PSD-95 was lower, but that the amount of NR2A that co-precipitated with αCaMKII was higher after CRF. Results are means ± SEMs. Control n = 8, CRF n = 8. *P < 0.01. CA1, cornu ammonis 1; CRF, chronic renal failure; NMDA, N-methyl-D-aspartate; PSD-95, postsynaptic density-95; αCaMKII, α isoform of calcium/calmodulin-dependent protein kinase II; SEM, standard error of mean.
Protein expressions of PSD-95 and αCaMKII in uremic hippocampi

It was examined whether levels of PSD-95 and αCaMKII were altered in the CA1 and CA3/DG subfields by CRF. Semi-quantitative immunoblotting demonstrated that CRF induced significant changes in the levels of both proteins in the CA1 subfield but not in the CA3/DG subfields. In the hippocampi of sham-operated control rats and CRF rats, immunoblotting for PSD-95 and αCaMKII revealed major strong bands at 95 kDa and 50 kDa, respectively (Fig. 3A). In the CA1 subfield, PSD-95 expression was significantly lower in CRF rats than in sham-operated controls (to $77 \pm 4.7\%$ of sham-operated controls, $P<0.01$, $n=8$). By contrast, αCaMKII expression in the CA1 subfield was significantly higher in CRF rats (to $157 \pm 10.3\%$ of sham-operated controls, $P<0.01$, $n=8$, Fig. 3B). Immunoblot analysis of the CA3/DG subfields did not reveal any changes in the expressions of PSD-95 or αCaMKII (Fig. 3A and B). α-Actin was used as internal control to confirm equal protein loadings.

Phosphorylations of GluR1 at ser831 and ser845 in the CA1 subfield

To determine whether CRF induces the phosphorylations of GluR1-ser831 and GluR1-ser845 in the uremic CA1 subfield, samples of sham-operated control and CRF CA1 tissues were immunoblotted with anti-GluR1-ser831 and anti-GluR1-ser845. In sham-operated control rats and CRF rats, immunoblotting for GluR1-ser831 and GluR1-ser845 revealed major strong bands at 132 kDa and 100 kDa, respectively (Fig. 5A). Furthermore, the expression of GluR1-ser831 in the uremic CA1 subfield was higher than in sham-operated controls (129 ± 3.5\% of sham-operated controls, $P<0.01$, $n=8$). However, the expression of GluR1-ser845 showed no significant change (Fig. 5B).

Discussion

Several studies have suggested that the frequency of cognitive disturbance is higher than that previously suspected and can be detected even in moderate chronic renal insufficiency [4,18]. In the present study, CRF was induced in rats by 5/6 nephrectomy and animals were then monitored for 7 weeks. In 2013, Fujisaki et al [19] followed rats for 4 weeks or 8 weeks to establish the long-term effects of uremia and found that serum creatinine and blood urea nitrogen concentrations in 5/6 nephrectomy mice were significantly higher than in control mice. However, they reported that no significant difference in all parameters between CKD-4W and CKD-8W. Hence, we have chosen the duration of follow-up to 7 weeks after surgery.

Uremia induced by CRF in the present study produced necrotic cell death and decreased neuronal marker protein (NueN) levels in the hippocampal CA1 subfield, but not in the CA3/DG subfields, indicating that CA1 is more vulnerable to neuronal cell death in CRF than CA3/DG. This proposition is generally in accord with observations of ischemic injury-induced changes in postsynaptic density, during which CA1 neurons are more prone to injury than CA3 or DG neurons [20]. Kesner et al [21] emphasized that CA1 subfield involved in spatial working memory in pattern separation, with CA3 subfield being more important in spatial working memory in pattern completion and in pattern association. Compromised renal function was found to present model CRF, in terms of increased plasma creatinine levels and decreased creatinine clearance. Similar to guanidine, guanidinosuccinic acid, and methylguanidine, creatinine is a guanidine compound and is thus considered a candidate uremic toxin. During uremia, the accumulation of neurotoxic metabolites may be important in the pathogenesis of uremic encephalopathy [22,23]. Brain guanidinosuccinic acid and creatinine concentrations, corresponding with intraperitoneal doses that induce clonic convulsions in mice, are similar to the concentrations found in the brains of uremic patients [23]. Therefore, increased levels of creatinine in plasma is a candidate factor responsible for the cell death of hippocampal CA1 subfield which may be closely
associated with dysfunction of spatial working memory in pattern separation. These toxins have been reported to increase the activation of NMDA receptors and to block GABA\_\alpha receptor ionophores [3]. The activation of NMDA receptors elicits Ca\^{2+} influx, potentially causing calcium-mediated neurotoxicity. Immunoprecipitation and immunoblotsing in the present study demonstrated that CRF was associated with significant changes in the expression of postsynaptic protein complexes. First, a selective decrease in the expression of PSD-95/NR2B synaptic complex in the uremic CA1 subfield was found. Wang et al [24] showed that PDZ1 domain of PSD-95 protects neurons from cerebral ischemia. However, in a rat model, preconditioning ischemia was found to attenuate transient hippocampal ischemic damage by inducing an increase of PSD-95 [25]. In addition, previous studies have shown that upregulation of NR2A and NR2B after chronic ethanol exposure is likely to be an adaptive response to prolonged inhibition of channel activity by ethanol because of no corresponding change in the amount of PSD-95 [26,27]. Therefore, PSD-95 downregulation by CRF may reduce the targeting of NR2B to synapses and may be related to the synaptic dysfunction. Secondly, it was found that NR2A/caMKII synaptic complex was upregulated in the uremic CA1 subfield. caMKII, which is essential for memory and learning acquisition, abundantly expressed in the hippocampus and is known to be involved in the regulation of the functional subunit composition of NMDA receptors in the postsynaptic zone [28,29]. Moreover, the regulation of caMKII is dependent on many factors, including PSD-95 [30]. Previous studies have suggested that NR2A is a key NMDA receptor subunit with respect to the modulation of caMKII binding to NMDA channel [23,24]. The upregulation of caMKII in the CA1 subfield suggests that NR2A-containing NMDA receptors may be increased in the synapse after CRF. Thus, our results suggest that activation of NMDA receptor resulted from CRF is closely associated with an alteration of the NR2 subunits of NMDA receptors at synapses.

Recently, Kim et al [31] reported that NMDA receptor activation has differential effects on the surface delivery of GluR1, and that these effects depend on the NR2A/NR2B subunit ratios of NMDA receptors at synapses. In the hippocampus, AMPA receptors are mainly composed of GluR1/GluR2 or GluR2/GluR3 dimers with a minority of GluR1 homomers [32]. Recently, it was demonstrated that the surface expression of GluR1 in mature neurons is supported by NR2A-containing NMDA receptors, but inhibited by NR2B-containing NMDA receptors [33]. Other studies have also indicated that in mature neurons, NR2B is associated with the inhibition of (rather than the activation of) the Ras-ERK signaling pathway, which drives GluR1 to synapses [31,34]. Thus, our results suggest that increases in NR2A-containing NMDA receptors after CRF induce Ras-ERK pathway activation and subsequent increase GluR1 delivery to synapses.

In a recent study, it was implied that the ser831 phosphorylation of GluR1 might partly underlie synaptic modification [35]. Furthermore, accumulating evidence suggests that GluR1 phosphorylation at ser831 is closely related to caMKII/NR2A synaptic complex during long-term potentiation (LTP) [36]. caMKII translocated to the postsynaptic density, formed sites to anchor GluR1, and phosphorylated GluR1 after being activated by Ca\^{2+} influx through NMDA receptor during LTP [36]. Thus, the observed increase in GluR1-ser831 phosphorylation, but not in GluR1-ser845 phosphorylation, in the uremic CA1 subfield suggests that activated caMKII translocated to the synaptic membranes and then activated GluR1-containing AMPA receptors via GluR1-ser831 phosphorylation. Furthermore, as elevated levels of activated GluR1-containing AMPA receptors directly affect Na\^{+} and Ca\^{2+} influx, and hence, increased Na\^{+} and Ca\^{2+} influxes through activated GluR1-containing AMPA receptors may play a role in the neuronal vulnerability of the CA1 subfield. Our results suggest that the activation of GluR1-containing AMPA receptors at synapses, caused by the upregulation of caMKII/NR2A synaptic complex, is directly responsible for neurotoxicity in CA1 neurons rather than NMDA-induced neurotoxicity.

Taken together, our findings show that uremia after CRF can induce neuronal cell death of hippocampal CA1 subfield which may result in cognitive impairment in the uremic rat brain. Upregulation of caMKII/NR2A synaptic complex and subsequent augmentation of the phosphorylated GluR1-containing AMPA receptor at synapses can be suggested as the hypothetical mechanism of hippocampal CA1 cell death.

**Conflict of interest**

The authors declare no conflict of interest.

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