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Uncaria rhynchophylla Upregulates the Expression of MIF and Cyclophilin A in Kainic Acid-Induced Epilepsy Rats: A Proteomic Analysis

Wan-Yu Lo,[*,//] Fuu-Jen Tsai,[†,//] Chung-Hsiang Liu,[**] Nou-Ying Tang,[†] Shan-Yu Su,[†,‡‡] Shinn-Zong Lin,[††,§§] Chun-Chung Chen,[§§] Woei-Cherng Shyu[‡,††] and Ching-Liang Hsieh[§,¶,‡‡]

[*]*Graduate Institute of Integrated Medicine*

[†]*School of Chinese Medicine*

[‡]*Graduate Institute of Immunology*

[§]*Graduate Institute of Acupuncture Science*

[¶]*Acupuncture Research Center*
China Medical University, Taichung, Taiwan

[//]*Department of Medical Research*

[**]*Department of Neurology*

[††]*Center for Neuropsychiatry*

[‡‡]*Department of Chinese Medicine*

[§§]*Department of Neurosurgery*
China Medical University Hospital, Taichung, Taiwan

Abstract: *Uncaria rhynchophylla* (Miq) Jack (UR) is a traditional Chinese herb and is used for the treatment of convulsive disorders, including epilepsy. Our previous study has shown that UR, as well as its major component *rhynchophylline* (RH), has an anticonvulsive effect and this effect is closely related to its scavenging activities of oxygen free radicals. The purpose of the present study was to investigate the effect of (UR) on the expression of proteins using a proteomics analysis in Sprague-Dawley (SD) rats with kainic acid (KA)-induced epileptic seizures. We profiled the differentially expressed proteins on two-dimensional electrophoresis (2-DE) maps derived from the frontal cortex and hippocampus of rat brain tissue 24 hours after KA-induced epileptic seizures. The results indicated that

Correspondence to: Dr. Ching-Liang Hsieh, Graduate Institute of Acupuncture Science, China Medical University. 91 Hsueh-Shih Road, Taichung, Taiwan. Tel: (+886) 4-2205-3366 (ext. 3600, 3609), Fax: (+886) 4-2203-5191, E-mail: clhsieh@mail.cmuh.org.tw
Co-correspondence to: Dr. Woei-Cherng Shyu, Center for Neuropsychiatry and Graduate Institute of Immunology, China Medical University Hospital, Taichung, Taiwan. Tel: (+886) 4-2205-2121 (ext. 7812), Fax: (+886) 4-2205-2121 (ext. 7810), E-mail: shyu9423@gmail.com

macrophage migration inhibitory factor (MIF) and cyclophilin A were under expressed in frontal cortex by an average of 0.19- and 0.23-fold, respectively. In the frontal cortex, MIF and cyclophilin A were significantly decreased in the KA group and these decreases were confirmed by the Western blots. However, in the hippocampus, only cyclophilin A was significantly decreased in the KA group. In addition, in real-time quantitative PCR (Q-PCR), MIF and cyclophilin A gene expressions were also significantly under expressed in the frontal cortex, and only the cyclophilin A gene was also significantly under expressed in the hippocampus in the KA group. These under expressions of MIF and cyclophilin A could be overcome by the treatment of UR and RH. In conclusion, the under expressions of MIF and cyclophilin A in the frontal cortex and hippocampus in KA-treated rats, which were overcome by both UR and UH treatment, suggesting that both MIF and cyclophilin A at least partly participate in the anticonvulsive effect of UR.

Keywords: *Uncaria rhynchophylla* (Miq) Jack; *Rhynchophylline*; Cyclophilin A; Macrophage Migration Inhibitory Factor (MIF); Proteomic Analysis; Kainic Acid-Induced Epileptic Seizure.

Introduction

Uncaria rhynchophylla (Miq) Jack (UR), a traditional Chinese herb, is used to treat convulsive disorders, such as epilepsy. Our previous studies had shown that UR has an anticonvulsive effect in kainic acid (KA)-induced epileptic model and these effects of UR have been shown to be related to the suppression of lipid peroxidation and to the scavenging of oxygen free radicals (Hsieh *et al.*, 1999a,b, 2009). UR has also been demonstrated to protect N-methyl-D-aspartate (NMDA)-induced neuronal damage and cytotoxicity in the hippocampus (Lee *et al.*, 2003a,b). Rhynchophylline (UH), a major component of UR, has been shown to defend against ischemia-induced neuronal damage via MNDA (Kang *et al.*, 2004). KA, an excitotoxic analog of glutamate isolated from the seaweed of Digentea simplex, induces epileptic behavioral changes in rats, including wet dog shakes, paw tremors, and facial myoclonia. Each type of seizure has its own characteristic electroencephalogram in rats (Hsieh *et al.*, 1999a,b, 2001). Several reports considered that KA-induced epileptic seizures are similar to psychomotor seizure in human because the main pathologic changes are in the hippocampus region of brain. Both the forebrain and hippocampus are high binding sites of KA (Schwob *et al.*, 1980; Ben-Ari, 1985).

Proteomics is a powerful tool to profile the differential expression of proteins in tissues to elucidate complex biological mechanisms (Liu *et al.*, 2008). The information offered by two-dimensional gel electrophoresis (2-DE) provides a context-based understanding of protein expressions (Krapfenbauer *et al.*, 2003; Kischel *et al.*, 2007). Currently, proteomics is widely employed in clinical research, particularly to reveal disease-relevant biomarkers (Krapfenbauer *et al.*, 2003; Kischel *et al.*, 2007; Liu *et al.*, 2008). Therefore, the purpose of this present study was to investigate the effect of (UR) on the expression of proteins using a proteomics analysis in Spragrue-Dawley (SD) rats with kainic acid

(KA)-induced epileptic seizures. We utilized clinical proteomic techniques to detect the UR- and UH-regulated proteins in KA-treated rats.

Materials and Methods

Animals

Male SD rats, weighing 200–300 g, were purchased from BioL ASCO Taiwan Co., Ltd. The rats were housed in standard iron cages in the animal center of the China medical university. The humidity level was controlled at $55 \pm 5\%$, and the rats were maintained on a 12-hours light-dark cycle at $22 \pm 2^{\circ}C$. All animal experiments were performed in *accordance with the Guidelines of the Chinese Society for Laboratory.*

The SD rats were randomly divided into 4 groups with 3 rats each as follows: (1) sham group, intra-peritoneal (i.p.) injection of 1.0 ml/kg phosphate buffer saline (PBS) only; (2) KA group, i.p. injection of KA (12 mg/kg; King Dom Co., Taipei) only; (3) UR group, i.p. injection of 1.0 g/kg/day UR, 2 days and 15 min, prior to the injection of KA.; and (4) RH group, the method was the same as that used in the UR group, except RH (0.25 mg/kg/day) was injected instead of UR. Behavioral observations and electroencephalogram (EEG) and electromyogram (EMG) recordings of rats were performed from 15 min prior to drug administration until 3 hours after KA administration. Finally, the rats were sacrificed; the frontal cortex and hippocampus were removed, separated, and stored at $-80^{\circ}C$ for proteomic analysis.

Preparation of the Epileptic Seizure Animal Model

One week prior to the EEG and EMG recordings, the head of the rat was fixed in a stereotactic apparatus under anesthesia by using chloral hydrate (400 mg/kg i.p.). The skull of the rat was exposed after the scalp was incised with a surgical knife from the midline, and then stainless steel screw electrodes were implanted over the bilateral sensorimotor cortices to serve as the recording electrodes. A reference electrode was placed on the frontal sinus for the EEG recordings. Bipolar electrical wires were placed on the neck muscle through the subcutaneous tissues for EMG recording. Finally, these electrodes were plugged into a connector, and then connected to an EEG and EMG recorder machine (MP100WSW; BIOPAC System, Inc., CA, USA) (Hsieh *et al.*, 1999a,b, 2001).

Preparation of UR and RH

UR was authenticated and extracted by the Koda Pharmaceutical Company (Taoyuan, Taiwan), which is a good manufacturing pharmaceutical (GMP) factory. Five hundred grams of crude UR was extracted twice with 3.5 and 2.5 L of distilled water. The total yield was 63.55 g (12.71%). The freeze-dried extracts of UR were identified by a high-performance liquid chromatography (HPLC) system (Interface D-700, Pump L-7100, UV-Vis Dector L-7420; Hitachi Instruments Service Co. Ltd., Ibaraki-ken, Japan) using RH

(Matsuura Yakugyo Co. Ltd., Japan) as a standard at the Koda Pharmaceutical Co. Ltd. Each gram of freeze-dried UR extract contained 0.22 mg of RH.

Protein Extractions

All tissues were minced into pieces of about 2–3 mm^3 and then homogenized with 0.5 ml of lysis buffer (8 M urea and 4% CHAPS) containing a protease inhibitor cocktail (Sigma, St. Louis, MO, USA) by the automated tissue homogenizer (MagNA Lyser; Roche Diagnostics, Penzberg, Germany). The lysates were centrifuged at 8000 rpm for 20 min at 4°C. The protein supernatants were collected and purified by acetone precipitation and the protein pellets were denatured with sample buffer (8 M urea, 4% CHAPS, 65 mM DTE, and 0.5% ampholytes). The final concentrations were measured using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA), according to the NenoDrop instructions.

Two-Dimensional Electrophoresis (2-DE)

The 2-DE method was carried out according to that reported by Lo *et al.* (2007). We used 250 μg of extracted proteins per sample for 2-DE separation. The rehydration solution containing the sample was then placed into a 17-cm immobilized pH gradient (pH 3–10) IPG strip (ReadyStrip IPG strip; Bio-Rad) and left overnight. First-dimension electrophoresis was carried out at 60 kVhr (PROTEAN IEF cell, Bio-Rad) at 20°C. IPG strips were equilibrated with 3 ml of equilibrium solution containing 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, a trace of bromophenol blue, and DTE (1% w/v) for 20 min, followed by a second equilibration for 20 min in the same equilibrium solution containing iodoacetamide (2.5% w/v) instead of DTE. The strips were then transferred to the top of 12% polyacrylamide gels and held in position with molten 0.5% agarose in running buffer containing 25 mM Tris, 0.192 M glycine, and 0.1% SDS. All gels were run at 16 mA/gel for 30 min, followed by 50 mA/ per gel for 4–5 hours. All experiments were done at least three times.

Detection of Protein Spots and Data Analysis

Gels were stained with silver nitrate and scanned by GS-800 imaging densitometer with PDQuest software (version 7.1.1; Bio-Rad). In order to compare the tissue protein distributions between the sham and the control groups (KA group), we made a screening for proteins that were under expressed (< 0.25-fold) in the control group (KA group) for advanced analysis by nanoelectrospray mass spectrometry (nano LC-MS/MS). All protein spots on the 2DE gels were quantified and compared using PDQuest software.

Enzyme Digestion and Analysis by Nano LC-MS/MS

Each protein spot (1$\sim$2 mm in diameter) that represented < 0.25-fold under expression in the control group was cut with a pipette tip and transferred into a microcentrifuge tube

(0.6 ml). The gel pieces were washed twice with 50 μl of 50% acetonitrile (ACN): 50% 200 mM ammonium bicarbonate for 5 min and shrunk with 100% acetonitrile until the gels turned white; the gels were then dried for 5 min in a speed vac. The gel pieces were rehydrated at room temperature in 15 μl of 50 mM ammonium bicarbonate (37°C for 4 min). An equivalent volume of trypsin (Promega, Madison, WI, USA) solution (20 ng/μl in 50 mM ammonium bicarbonate) was then added and the gel pieces were incubated at 37°C for 4 hours. After digestion, the gel pieces were vortexed and spun down; the resulting supernatant peptide solutions were stored at −80°C until mass analysis.

All analyses were performed using an Ultimate capillary LC system (LC Packings, Amsterdam, The Netherlands) coupled to a QSTARXL quadrupole-time of flight (Q-TOF) mass spectrometer (Applied Biosystem/MDS Sciex, Foster City, CA, USA). The nanoscale capillary LC separation was performed in an RP C18 column. The product ion spectra generated by nanoscale capillary LC-MS/MS (nanoLC-MS/MS) were scanned for exact matches using the MASCOT search program. "Rat" taxonomy restriction was used and the mass tolerance of both precursor ion and fragment ions was set at 0.3 Da.

Real-Time Quantitative PCR (Q-PCR)

Total RNA was reverse-transcribed into cDNA using an oligo (dT) 20 primer and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). The specific gene primers (Supplemental data 1) for real-time PCR were designed using the Beacon Designer 2.1 program (Premier Biosoft International, Palo Alto, CA, USA). The PCR reaction (25 μl) containing forward and reverse primers was run for 40 amplification cycles on an Applied Biosystems 7300 Real-Time PCR system (Applied Biosystems). Cycling conditions were 50°C for 2 min, 95°C for 10 min, 95°C for 15 sec, and 60°C for 1 min. Relative transcript quantities were calculated using the $\Delta\Delta C_t$ method with β-actin as the reference gene amplified from the samples. Relative expression (fold changes) was determined using the comparative C_T method. All reactions were run in triplicate.

Western Blots

The frontal cortex and hippocampus were homogenized in lysis buffer and centrifuged at 12,000 rpm at 4°C for 4 min. The supernatants were stored at −80°C until the Western blotting analysis. The protein extracts (20 μg) were separated by 10% SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were probed with anti-macrophage migration inhibitory factor (MIF; Santa Cruz Biotechnology, Inc., CA, USA) and anti-cyclophilin A (Cell Signaling, Beverly, MA, USA) antibodies. The bound antibodies were detected by chemiluminescence (ECL System, Amersham, Buckinghamshire, UK) and autoradiography.

Statistical Analysis

All results are expressed as the mean ± SE. The relative levels of expression of tissue samples in the sham, control, UR, and RH groups were compared using a paired t-test.

Two-sided $p < 0.05$ was considered as significant. All analyses were performed with SAS 9.0 software (SAS, Inc., Cary, NC, USA).

Results

KA-Induced Epileptic Seizures in SD Rats

We observed KA-induced behavioral seizures, and EEG and EMG changes to confirm the development of epileptic seizures in SD rats. KA-induced-epileptic seizure includes wet dog shakes, paw tremor, and facial myoclonia. The characteristic EEG activity was noted in each type of seizure (Fig. 1).

2-DE Analysis of the Frontal Cortex and Hippocampus

Figures 2A and 2B illustrate the representative images of the frontal cortex and hippocampus fractions respectively from sham and control groups separated by 2-DE and stained with silver. The total protein extraction rates did not differ significantly between the two groups. Fully automated spot detection and quantification were also performed using PDQuest software, followed by automated image-to-image matching and statistical analysis in the experiment. The averages of 650–835 spots were detected across all gels. The abundance ratios of proteins in the frontal cortex and hippocampus in the sham and the control groups were calculated from the 2-DE gels. The distinct spots showed a reduction (< 0.25-fold) in the KA group and were assigned a number for advance nano-LC-MS/MS analysis. PDQuest analysis indicated that the expression of spots 1 and 2 decreased by an average of 0.19- and 0.23-fold in the 2-DE maps of the frontal cortex in the KA group, respectively (Table 1 and Fig. 2A). Unexpectedly, spots 1 and 2 showed weak reductions by an average of 0.87- and 0.63-fold in 2-DE analysis in the KA group in the hippocampus (Fig. 2B).

Spots 1 and 2 were matched and identified as MIF and cyclophilin A by nano-LC- MS/MS analysis. The identification of MIF was substantiated by MS/MS data on the following three tryptic peptides: PMFIVNTNVPR (Ions score 91), IGGAQNR (Ions score 34), and IGGAQNRNYSKLLCGLLSDRLHSPDR (Ions score 111). These peptides spanned nearly 35% of MIF's linear amino acid sequence (Score: 506). Cyclophilin A was identified based on MS/MS data from five tryptic peptides with the following sequences: VNPTVFFDITADGEPLGRVCFELFADKVPK (Ions score 66); IIPGFMCQGGDFTR (Ions score 41); HTGPGILSMANAGPNTNGSQFFICTAKTEWLDGK (Ions score 103); HVVFGKVKEGMSIVEAMER (Ions score 41); and FGSR (Ions score 26). These peptides spanned nearly 61% of cyclophilin A's linear sequence (Score: 1345). Table 1 lists a summary of protein identification, sequence coverage, and characterizations for MIF and cyclophilin A.

Confirmation of Decreased MIF and Cyclophilin A by Western Blots

Western blotting with antibodies recognizing rat MIF and cyclophilin A revealed prominent bands of 12 and 18 kDa, respectively (Figs. 3A and 3B). The levels of protein

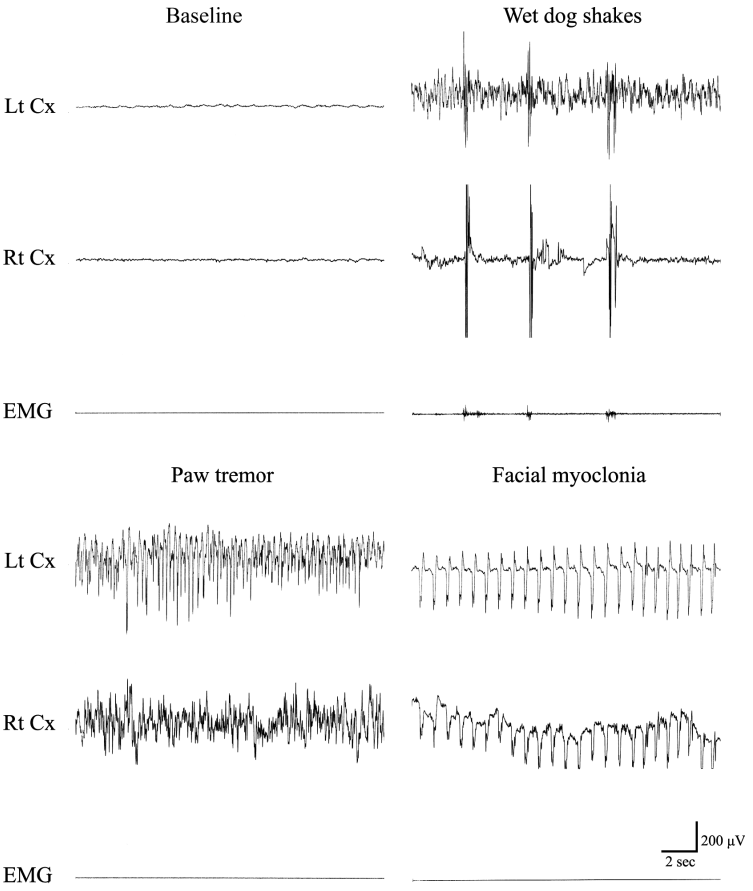
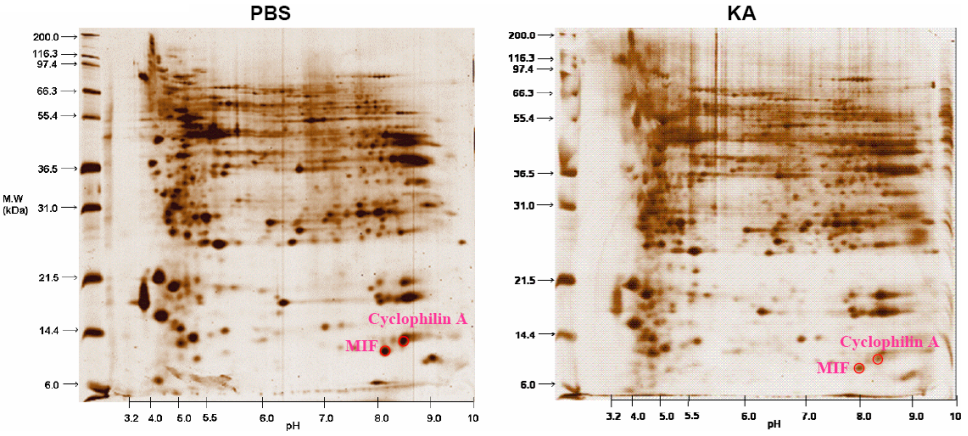


Figure 1. EEG and EMG changes in kainic acid (KA)-induced epileptic seizures SD rats. Lt Cx: EEG recordings in the left sensorimotor cortex; Rt Cx: EEG recordings in the right sensorimotor cortex; EMG: EMG recordings in the neck muscle; Baseline: baseline EEG activities in rat during awake state (left upper); Wet dog shakes: KA-induced wet dog shakes with intermittent polyspike-like artifact (right upper); Paw tremor: KA-induced paw tremor with continuous spike EEG activities (left lower); Facial myoclonia: KA-induced facial myoclonia with continuous sharp EEG activities (right lower).

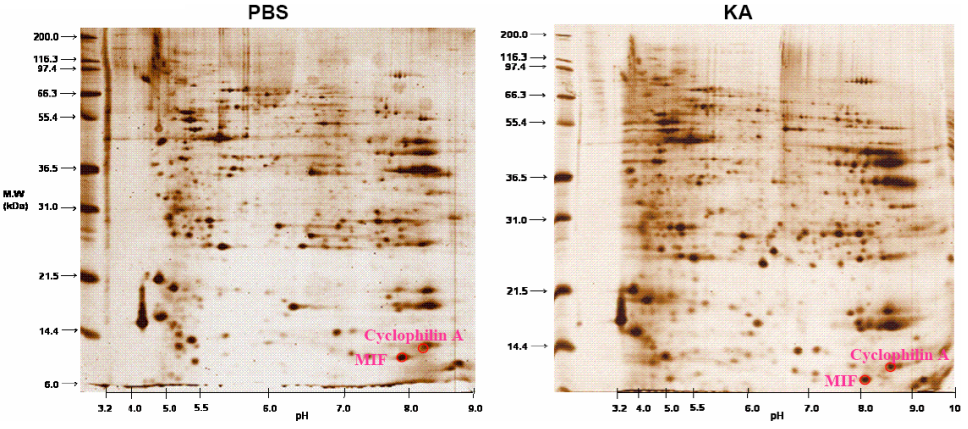
expression of MIF and cyclophilin A were decreased in the frontal cortex after KA administration; the results were consistent with those were found on 2-DE (Fig. 3A). The decreased expressions of MIF and cyclophilin A were also observed in the hippocampus (Fig. 3B). It demonstrated that MIF and cyclophilin A were significantly decreased in both the frontal cortex and hippocampus after KA administration.

KA Inhibits Gene Expression of MIF and Cyclophilin A

In the frontal cortex, the results showed that the MIF and cyclophilin A gene expressions were down-regulated significantly by an average of 25% and 22% respectively in each



(A) Frontal cortex.



(B) Hippocampus.

Figure 2. The 2-DE maps of the frontal cortex (A) and hippocampus (B) in KA-treated experiments. The two spots (Nos. 1 and 2) were depressed in the frontal cortex in the control group and the protein identified by nano LC-MS/MS. The two spots were identified to be MIF and cyclophilin A, respectively. PBS: sham group; KA: kainic acid-treated group.

tissue RNA of the KA group. In the hippocampus, only the cyclophilin A gene was down-regulated significantly by 26% in the KA group. In other words, the *MIF* gene was only significantly under expressed in the frontal cortex approximately 25% after KA administration and there were no significant alterations in transcription level in the hippocampus (Fig. 4).

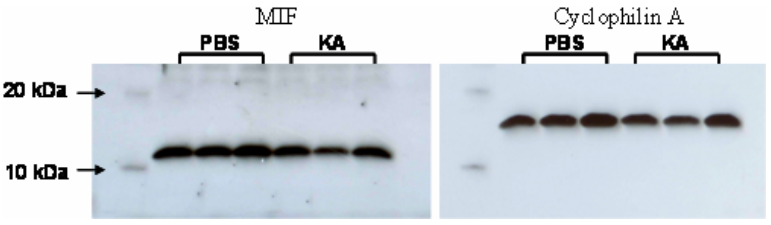
Overcoming the Decreased MIF and Cyclophilin A Proteins by UR and RH Treatments

Our previous studies have demonstrated that UR has anticonvulsive effects in KA-induced epilepsy rats. Therefore, we carried out the Western blots to detect whether UR

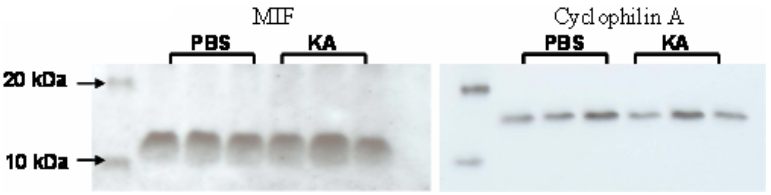
Table 1. Characterization of MIF and Cyclophilin A in 2-DE Maps and Database Searching

No.	Accession No.	Protein ID	Synonyms	Theoretical Mr(kDa)/ PI	Sequences Coverage	Characterization
1	P30904	MIF	Phenylpyruvate tautomerase, Glutathione-binding 13 kDa protein	12.5/6.8	35%	The expression of MIF at sites of inflammation suggests a role for the mediator in regulating the function of macrophages in host defense
2	P10111	Cyclophilin A	Peptidyl-prolyl cis-trans isomerase A (PPIA), Rotamase A, Cyclosporin A-binding rotein, p31, p1B15	17.9/8.3	61%	Cyclophilin A accelerates the folding of proteins and catalyzes the cis-trans isomerization of proline imidic peptide bonds in oligopeptides

Note: MIF: macrophage migration inhibitory factor.



(A) Frontal cortex.



(B) Hippocampus.

Figure 3. The protein expressions of MIF and cyclophilin A in the frontal cortex (A) and hippocampus (B) from the sham and KA groups, respectively. Twenty micrograms of each sample was separated on 10% SDS-PAGE. PBS: sham group; KA: kainic acid-treated group.

and its component RH treatments also affect MIF and cyclophilin A protein expressions in frontal cortex and hippocampus lysates of KA-induced epilepsy rats. In this study, rat MIF and cyclophilin A proteins showed prominent bands of 12.5 kDa and 18 kDa respectively in the frontal cortex and hippocampus lysates from sham (normal), control,

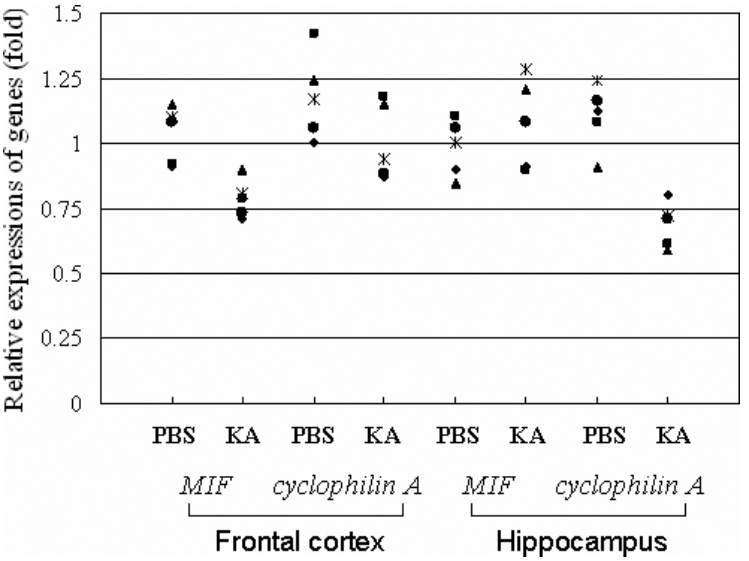
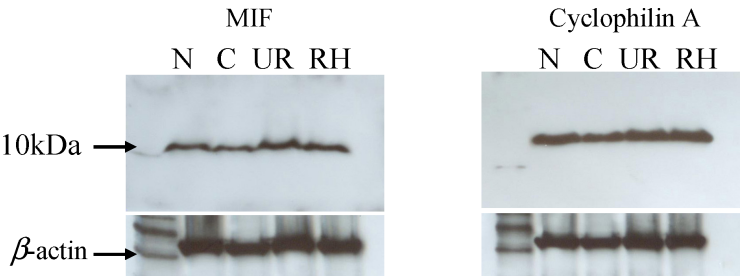


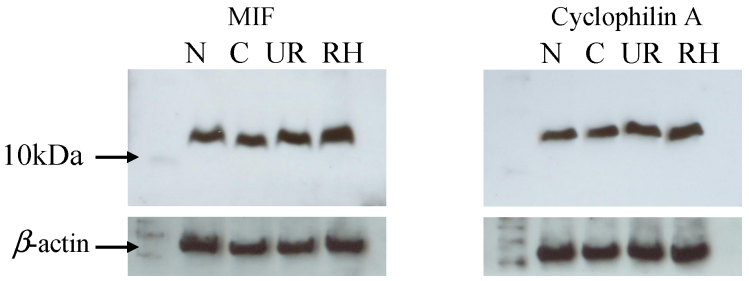
Figure 4. The relative expressions of the MIF and cyclophilin A genes were identified by real time Q-PCR in the frontal cortex and hippocampus from the sham and KA groups. Data show the relative expression of each sample in the PBS and KA group (n = 5). PBS: sham group; KA: kainic acid-treated group.

UR treated and RH treated groups, (Figs. 5A and 5B). Both in the frontal cortex and hippocampus, MIF and cyclophilin A were significantly decreased in the control (KA group) and overcome by UR and RH treatments. In the frontal cortex, MIF showed an average increase of 3.1- and 2.75-fold and cyclophilin A showed an average increase of 2.08- and 1.83-fold in the UR and RH groups, which were significant compared to the control (KA group) (Fig. 5C). Similarly, the expression of MIF and cyclophilin A also

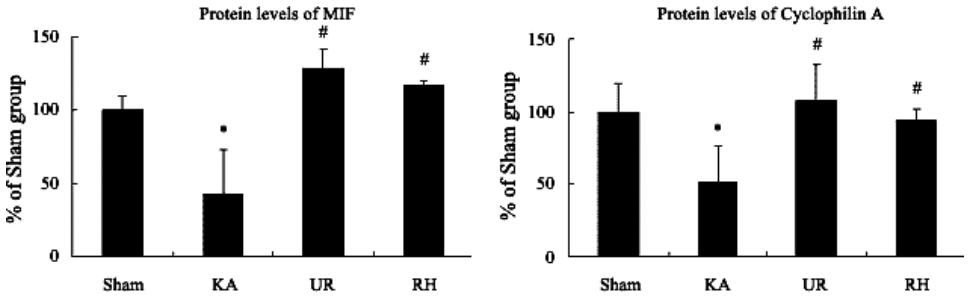


(A) Frontal cortex.

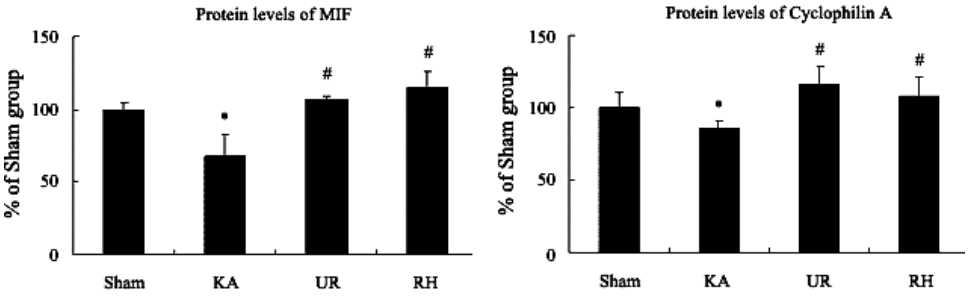
Figure 5. The Western blots of MIF and cyclophilin A in the frontal cortex (A) and hippocampus (B) from sham, control, UR, and RH groups. Quantification of the levels of protein expression is shown in (C) and (D). *Statistically significantly different from the individual sham group in KA groups based on student's t-test ($p < 0.05$). #Statistically different from the individual KA group in the UR and RH groups based on student's t-test ($p < 0.05$). N: sham group with PBS treatment; C: KA group with kainic acid treatment; UR: UR group with *Uncaria rhynchophylla* treatment; RH: RH group with *rhynchophylline* treatment.



(B) Hippocampus.



(C) Frontal cortex.



(D) Hippocampus.

Fig. 5. (*Continued*)

showed an average increase of 1.57- and 1.69-fold, and 1.35- and 1.26-fold in the UR and RH groups, respectively, in the control (KA group) of hippocampus lysates. In either the frontal cortex or hippocampus, the results indicated that both MIF and cyclophilin A levels in the UR and RH groups were significantly greater than the control (KA group). These results also demonstrated the decrease in MIF and cyclophilin A could overcome by UR and RH (Fig. 5D).

Discussion

The results of the present study indicated that both MIF and cyclophilin A were under expressed in the frontal cortex, and cyclophilin A was under expressed in the

hippocampus in KA-treated rats, whereas these under-expressions of MIF and cyclophilin A could be overcome by the treatment of UR and UH. In our previous study, UR has been shown to have anticonvulsive effect in KA-induced epileptic seizure rats (Hsieh *et al.*, 1999a,b). Therefore, our data suggest that MIF and cyclophilin A are at least in part involved in anticonvulsive mechanism of UR. 2-DE analysis is a powerful tool for separating complex protein mixtures from tissues, cells, and subcellular fractions. In 2004, Eun *et al.* (2004) demonstrated a link between the pathogenesis of epilepsy and superoxidase dismutase from epileptic cerebral cortices of patients by 2-DE comparisons and quantitative proteome analysis. Recently, some studies have also focused on the differentially expressed proteins in the pilocarpine model of convulsive status epilepticus (CSE) and temporal lobe epilepsy (TLE) by other proteomic analyses (Greene *et al.*, 2007; Liu *et al.*, 2008).

MIF is a small protein (12.5 kDa) found in immune tissues and blood, and functions as a cytokine in immune responses (Fingerle-Rowson and Bucala, 2001). MIF is also expressed in the CNS, including neurons within the hypothalamus (Bacher *et al.*, 1998). Previous studies have demonstrated that MIF is produced intracellularly in neurons cultured from the hypothalamus and brain stem of normotensive SD rats and in the rat paraventricular nucleus (Li *et al.*, 2006). Furthermore, increased levels of intracellular MIF have been shown to reduce the AT1R- mediated chronotropic actions in SD and Wistar–Kyoto (WKY) rats (Li *et al.*, 2006). Collectively, these studies suggest that MIF serves as a counter-regulator of normal neuronal actions. In the current study, the acute reduction of MIF protein and gene expression were also observed in the frontal cortex in SD rats exposed to KA-induced epileptic seizures (KA group), although a reduction in the expression of the MIF gene was not observed in the hippocampus. Therefore, it is possible that MIF gene regulation contributes as a biomarker of epileptic seizure only within the frontal cortex in the KA-induced epileptic seizure model. Other data have extended the role of this cytokine and indicated an involvement in neuroendocrine-immunologic interactions due to its localization in the pituitary and its release from the anterior pituitary cells after endotoxemia (Bucala, 1996). Thus, MIF may not only represent an important factor in the immune system, but may also be expressed and become functional in the CNS. This assumption is also supported by studies indicating an involvement of MIF in neuroendocrine regulation and neurologic disease (Yoshimoto *et al.*, 1997; Bacher *et al.*, 1998).

Cyclophilins are a family of phylogenetically-conserved proteins found from prokaryotes to humans. This family of proteins has peptidyl-prolyl isomerase activity *in vitro*, indicating that these proteins can influence the conformation of other proteins. However, the exact functions are largely unknown (Fischer *et al.*, 1989). A total of 15 cyclophilin proteins are expressed in human. Cyclophilin A is an abundant, ubiquitously-expressed protein that was originally discovered as an intracellular ligand of the immunosuppressive agent, cyclosporin A (CsA) (Handschumacher *et al.*, 1984). The cyclophilin A-CsA complex has been shown to mediate immunosuppression by inhibiting calcineurin, a phosphatase for T cell activation (Liu *et al.*, 1991). Montague *et al.* (1994) reported that cyclophilin A possesses latent apoptosis-related DNase activity. Cyclophilin A has also been shown to participate in excitotoxin-induced apoptosis (Capano *et al.*, 2002) and to

interact with dynein (Galigniana *et al.*, 2004), the antioxidant protein Aop1 (Jäschke *et al.*, 1998), and apoptosis-inducing factor (AIF) (Candé *et al.*, 2004). Data from 2-DE and Western blots in this study indicate that cyclophilin A expression is only significantly reduced in the frontal cortex, but not in the hippocampus. It suggests that cyclophilin A expression is a suitable index of KA-induced epileptic seizures. Interestingly, we also found significant differences in the gene level of cyclophilin A in both the frontal cortex and hippocampus in KA-induced epileptic seizure rats, but additional evidence is needed to explain the underlying mechanism.

Furthermore, we also demonstrated that the decreased MIF and cyclophilin A expressions were overcome by UR and RH in KA-treated rats. Quantization the levels of MIF and cyclophilin A by Western blots has shown that RH is as effective as UR in rats exposed to KA-induced epileptic seizures. This result suggests that RH is not only a major component of UR but also an active molecule (Kang *et al.*, 2004; Matsumoto *et al.*, 2005).

Briefly, we observed the under expressions of MIF and cyclophilin A in the frontal cortex and hippocampus in KA-treated rats. This decrease was overcome by UR and UH treatment, suggesting that both MIF and cyclophilin A at least partly participate in the anticonvulsive effect of UR.

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References

Bacher, M., A. Meinhardt, H.Y. Lan, F.S. Dhabhar, W. Mu, C.N. Metz, J.A. Chesney, D. Gemsa, T. Donnelly, R.C. Atkins and R. Bucala. MIF expression in the rat brain: implications for neuronal function. *Mol. Med.* 4: 217–230, 1998.

Ben-Ari, Y. Limbic seizure and brain damage produced by kainic acid: mechanisms and relevance to human temporal lobe epilepsy. *Neuroscience* 14: 375–403, 1985.

Bucala, R. MIF rediscovered: cytokine, pituitary hormone, and glucocorticoid-induced regulator of the immune response. *FASEB J.* 10: 1607–1613, 1996.

Candé, C., N. Vahsen, I. Kouranti, E. Schmitt, E. Daugas, C. Spahr, J. Luban, R.T. Kroemer, F. Giordanetto, C. Garrido, J.M. Penninger and G. Kroemer. AIF and cyclophilin A cooperate in apoptosis-associated chromatinolysis. *Oncogene* 23: 1514–1521, 2004.

Capano, M., S. Virji and M. Crompton. Cyclophilin-A is involved in excitotoxin-induced caspase activation in rat neuronal B50 cells. *Biochem. J.* 363: 29–36, 2002.

Eun, J.P., H.Y. Choi and Y.G. Kwak. Proteomic analysis of human cerebral cortex in epileptic patients. *Exp. Mol. Med.* 36: 185–191, 2004.

Fingerle-Rowson, G.R. and R. Bucala. Neuroendocrine properties of macrophage migration inhibitory factor (MIF). *Immunol. Cell Biol.* 79: 368–375, 2001.

Fischer, G., B. Wittmann-Liebold, K. Lang, T. Kiefhaber and F.X. Schmid. Cyclophilin and peptidyl-prolyl cis-trans isomerase are probably identical proteins. *Nature* 337: 476–478, 1989.

Galigniana, M.D., Y. Morishima, P.A. Gallay and W.B. Pratt. Cyclophilin-A is bound through its peptidylprolyl isomerase domain to the cytoplasmic dynein motor protein complex. *J. Biol. Chem.* 279: 55754–55759, 2004.

Greene, N.D.E., A. Bamidele, M. Choy, S.C.P. de Castro, R. Wai, K.Y. Leung, S. Begum, D.G. Gadian, R.C. Scott and M.F. Lythgoe. Proteome changes associated with hippocampal MRI abnormalities in the lithium pilocarpine-induced model of convulsive status epilepticus. *Proteomics* 7: 1336–1344, 2007.

Handschumacher, R.E., M.W. Harding, J. Rice, R.J. Drugge and D.W. Speicher. Cyclophilin: a specific cytosolic binding protein for cyclosporin A. *Science* 226: 544–547, 1984.

Hsieh, C.L., M.F. Chen, T.C. Li, S.C. Li, N.Y. Tang, C.T. Hsieh, C.Z. Pon and J.G. Lin. Anticonvulsant effect of *Uncaria rhynchophylla* (Miq) Jack. in rats with kainic acid-induced epileptic seizure. *Am. J. Chin. Med.* 27: 257–264, 1999a.

Hsieh, C.L., N.Y. Tang, S.Y. Chiang, C.T. Hsieh and J.G. Lin. Anticonvulsive and free radical scavenging actions of two herbs, *Uncaria rhynchophylla* (Miq) Jack and *Gastrodia elata* Bl., in kainic acid-treated rats. *Life Sci*. 65: 2071–2082, 1999b.

Hsieh, C.L., S.Y. Chiang, K.S. Cheng, Y.H. Lin, N.Y. Tang, C.J. Lee, C.Z. Pon and C.T. Hsieh. Anticonvulsive and free radical scavenging activities of *Gastrodia elata* Bl. in kainic acid-treated rats. *Am. J. Chin. Med.* 29: 331–341, 2001.

Hsieh, C.L., T.Y. Ho, S.Y. Su, W.Y. Lo, C.H. Liu and N.Y. Tang. *Uncaria rhynchophylla* and Rhynchophylline inhibit c-Jun N-terminal kinase phosphorylation and nuclear factor-kappaB activity in kainic acid-treated rats. *Am. J. Chin. Med.* 37: 351–360, 2009.

Jäschke, A., H. Mi and M. Tropschug. Human T cell cyclophilin 18 binds to thiol-specific antioxidant protein Aop1 and stimulates its activity. *J. Mol. Biol.* 277: 763–769, 1998.

Kang, T.H., Y. Murakami, H. Takayama, M. Kitajima, N. Aimi, H. Watanabe and K. Matsumoto. Protective effect of rhynchophylline and isorhynchophylline on *in vitro* ischemia-induced neuronal damage in the hippocampus: putative neurotransmitter receptors involved in their action. *Life Sci.* 76: 331–343, 2004.

Kischel, P., D. Waltregny and V. Castronovo. Identification of accessible human cancer biomarkers using *ex vivo* chemical proteomic strategies. *Expert Rev. Proteomics* 4: 727–739, 2007.

Krapfenbauer, K., E. Engidawork, N. Cairns, M. Fountoulakis and G. Lubec. Aberrant expression of peroxiredoxin subtypes in neurodegenerative disorders. *Brain Res.* 967: 152–160, 2003.

Lee, J., D. Son, P. Lee, D.K. Kim, M.C. Shin, M.H. Jang, C.J. Kim, Y.S. Kim, S.Y. Kim and H. Kim. Protective effect of methanol extract of *Uncaria rhynchophylla* against excitotoxicity induced by N-methyl-D-aspartate in rat hippocampus. *J. Pharmacol. Sci.* 92: 70–73, 2003a.

Lee, J., D. Son, P. Lee, S.Y. Kim, H. Kim, C.J. Kim and E. Lim. Alkaloid fraction of *Uncaria rhynchophylla* protects against N-methyl-D-aspartate-induced apoptosis in rat hippocampal slices. *Neurosci. Lett.* 348: 51–55, 2003b.

Li, H., Y. Gao, C.D. Freire, M.K. Raizada, G.M. Toney and C. Sumners. Macrophage migration inhibitory factor in the PVN attenuates the central pressor and dipsogenic actions of angiotensin II. *FASEB J.* 20: E1146–E1156, 2006.

Liu, J., J.D. Farmer Jr., W.S. Lane, J. Friedman, I. Weissman and S.L. Schreiber. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* 66: 807–815, 1991.

Liu, X.Y., J.L. Yang, L.J. Chen, Y. Zhang, M.L. Yang, Y.Y. Wu, F.Q. Li, M.H. Tang, S.F. Liang and Y.Q. Wei. Comparative proteomics and correlated signaling network of rat hippocampus in the pilocarpine model of temporal lobe epilepsy. *Proteomics* 8: 582–603, 2008.

Lo, W.Y., C.C. Lai, C.H. Hua, M.H. Tsai, S.Y. Huang, C.H. Tsai and F.J. Tsai. S100A8 is identified as a biomarker of HPV 18-infected oral squamous cell carcinomas by suppression subtraction hybridization, clinical proteomics analysis, and immunohistochemistry staining. *J. Proteome Res.* 6: 2143–2151, 2007.

Matsumoto, K., R. Morishige, Y. Murakami, M. Tohda, H. Takayama, I. Sakakibara and H. Watanabe. Suppressive effects of isorhynchophylline on 5-HT_{2A} receptor function in the brain: behavioural and electrophysiological studies. *Eur. J. Pharmacol*. 517: 191–199, 2005.

Montague, J.W., M.L. Gaido, C. Frye and J.A. Cidlowski. A calcium-dependent nuclease from apoptotic rat thymocytes is homologous with cyclophilin. Recombinant cyclophilins A, B, and C have nuclease activity. *J. Biol. Chem.* 269: 18877–18880, 1994.

Schwob, J.E., T. Fuller, J.L. Price and J.W. Olney. Widespread patterns of neuronal damage following systemic or intracerebral injections of kainic acid: a histological study. *Neuroscience* 5: 991–1014, 1980.

Yoshimoto, T., J. Nishihira, M. Tada, K. Houkin and H. Abe. Induction of macrophage migration inhibitory factor messenger ribonucleic acid in rat forebrain by reperfusion. *Neurosurgery* 41: 648–653, 1997.

Supplement Data 1 Primers for Real-Time Quantitative PCR

Primer Names	Primer Sequences (5′–3′)	Amplifiers (bp)
MIF-F	CCCAGAACCGCAACTACAG	103
MIF-R	GCAGCGTTCATGTCGTAATAGT	103
Cyclophilin A-F	GACGCCACTGTCGCTTTT	114
Cyclophilin A-R	CTGCAAACAGCTCGAAGGA	114