Original Paper

Extracellular HMGB1 Modulates Glutamate Metabolism Associated with Kainic Acid-Induced Epilepsy-Like Hyperactivity in Primary Rat Neural Cells

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Key Words

Epilepsy • HMGB1 • Kainic acid • Primary rat neural cells • Glutamate • GABA

Abstract

Background/Aims: Neuroinflammatory processes have been implicated in the pathophysiology of seizure/epilepsy. High mobility group box 1 (HMGB1), a non-histone DNA binding protein, behaves like an inflammatory cytokine in response to epileptogenic insults. Kainic acid (KA) is an excitotoxic reagent commonly used to induce epilepsy in rodents. However, the molecular mechanism by which KA-induced HMGB1 affords the initiation of epilepsy, especially the role of extracellular HMGB1 in neurotransmitter expression, remains to be elucidated. **Methods:** Experimental early stage of epilepsy-related hyperexcitability was induced in primary rat neural cells (PRNCs) by KA administration. We measured the localization of HMGB1, cell viability, mitochondrial activity, and expression level of glutamate metabolism-associated enzymes. **Results:** KA induced the translocation of HMGB1 from nucleus to cytosol, and its release from the neural cells. The translocation is associated with post-translational modifications. An increase in extracellular HMGB1 decreased PRNC cell viability and mitochondrial activity, downregulated expression of glutamate decarboxylase67 (GAD67) and glutamate dehydrogenase (GLUD1/2), and increased intracellular glutamate concentration and major histocompatibility complex II (MHC II) level. **Conclusions:** That a surge in extracellular HMGB1 approximated seizure initiation suggests a key pathophysiological contribution of HMGB1 to the onset of epilepsy-related hyperexcitability.

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Introduction

Epilepsy is a debilitating neurological disorder affecting approximately 2% of the world's population. Over three million Americans suffer from some form of epilepsy, with mesial temporal lobe epilepsy (TLE) being the most common, arising from the temporal lobe structures of amygdala, hippocampus, and parahippocampal gyrus [1]. Despite the availability of many antiepileptic drugs (AEDs), greater than 30% of TLE patients will have seizures refractory to standard therapeutics, highlighting the need for more effective therapies [2].

High mobility group box 1 (HMGB1) belongs to a damage-associated-molecular-patterns (DAMP) family [3], is a highly conserved non-histone nuclear protein, and contributes to the architecture of chromatin DNA [4]. HMGB1 is a promiscuous sensor driving a pathogenic inflammatory mediator such as septic shock [5, 6], ischemia [7-10], traumatic brain injury [11], epilepsy [12], Parkinson's disease [13], Alzheimer's disease [14], pancreatitis [15], and other neurodegenerative disorder diseases [16]. HMGB1 induces inflammation mainly through receptor for advanced glycation end products (RAGE) and Toll-like receptor 4 [12, 17-19].

Unilateral injection of kainic acid (KA), an agonist to the excitatory neurotransmitter glutamate, into the rodent brain induces epileptic seizures, and granule cell dispersion [20]. Seizures are the results of an imbalance between excitatory and inhibitory neuronal activities. Increased glutamate release into synaptic clefts, in part, leads to excitotoxic lesion-induced epilepsy. KA promotes neurotoxicity involving epileptogenic effects via the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid and kainate receptors in modulating the neurotransmitter glutamate [21]. A high concentration of glutamate, resulting from hyperexcitation of the neuronal network, is thought to play a key role in the initiation and spread of seizures. Interestingly, KA has been reported to induce seizures in animals coincident with a significant increment in HMGB1 concentration in the hippocampus [22]. Although HMGB1 translocation has been shown to be tightly regulated by post-translation modifications, in particular altered expression of glutamate metabolism-associated enzymes, such molecular mechanism underlying KA-induced HMGB1 upregulation remains unexplored.

In this study, using our recently reported cell culture model of early stage epilepsy-related hyperexcitability involving KA treatment of primary rat neural cells (PRNCs) [23], we examined HMGB1 translocation (nuclear to cytosol to extracellular space) and the expression levels of glutamate metabolism-associated enzymes. The present results demonstrated that aberrant extracellular HMGB1 may contribute to the disease pathophysiology of epilepsy-related hyperexcitability.

Materials and Methods

Cell culture

Primary rat neural cells (PRNCs) were obtained from BrainBit (E18 rat cortex) and grown in culture as we previously reported [24]. Cells (4×10^4 cells/well) were suspended in 200 μ l Neural Medium (NbActive 4, BrainBit NB4-500) containing 2 mM *L*-glutamine and 2% B27 in the absence of antibiotics and grown in poly-*L*-lysine-coated 96-well plates (BD 354516) at 37°C in humidified atmosphere containing 5% CO₂. We were able to consistently grow PRNCs consisting of 40% neurons and 60% astrocytes [24]. After 3 days in culture, approximately 60% cell confluence was achieved. Next, PRNCs (8×10^4 cell/well) were cultured in 400 μ l neural medium containing 2 mM *L*-glutamine and 2% B27 in the absence of antibiotics in poly-*L*-lysine 8-chamber (BD 354632) for 3 days at 37°C. Thereafter, the cells were exposed to 10 μ M KA alone, 1 μ g/ml HMGB1 (Chimerigen Laboratories, NP 0370951.1), 10 μ M KA in the presence of 1 μ g/ml HMGB1, 1 μ g/ml HMGB1 in the presence of 1 μ g/ml anti HMGB1 antibody, or in the absence of reagent (control) for 3 days at 37°C.

Immunocytochemistry

Following treatments mentioned above, PRNCs were fixed in 4% paraformaldehyde [24]. The cells were washed 5 times for 10 min in phosphate saline buffer (PBS) containing 0.1% Tween-20 (PBST), and then were blocked by 5% normal goat serum (Invitrogen 50062Z) in PBST for 1 h at room temperature (RT). Primary antibodies, anti-HMGB1 rabbit monoclonal antibody (Abcam, ab79823, 1/500) and anti-microtubule-associated protein 2 (MAP2) mouse monoclonal antibody (Abcam, ab11267, 1/10,000) were used. The cells were incubated overnight at 4°C with primary antibody with 5% normal goat serum. The cells were washed 5 times for 10 min in PBST and then soaked in 5% normal goat serum in PBST containing corresponding secondary antibodies, goat anti-rabbit IgG-Alexa 488 (green, Invitrogen A11008, 1/1,000) and goat anti-mouse IgG-Alexa 594 (red, Invitrogen A11005, 1/1,000) for 90 min in dark. Finally, cells were washed 5 times for 10 min in PBST and 3 times for 5 min in PBS, then processed for Hoechst 33258 (Sigma B2883) for 30 min, washed in PBS, and cover-slipped with Fluoromount. Immunofluorescent images were visualized using confocal microscope (Olympus FV1000). Control experiments were performed with the omission of the primary antibodies yielding negative results. HMGB1 translocation from nucleus to cytosol fraction was assessed by measuring the area of the cells with visible cytoplasmic HMGB1 staining from at least five randomly selected microscopies field (400×, cell count approximately 50) using ImageJ [23].

Measurement of extracellular HMGB1 concentration

After PRNC treatments as detailed above, the culture medium was centrifuged at 3000 g, 4°C for 15 min, and the supernatant was processed for HMGB1 expression using ELISA kit (amin416082, Antibody, Atlanta, GA, USA) and measured the absorbance at 450 nm on a Synergy HT plate reader (Bio-Tex).

Measurement of cell viability

Measurement of cell viability was performed by both fluorescent live/dead cell assay and trypan blue exclusion method [25]. A two-color fluorescence cell viability assay was performed by calcein acetoxymethyl ester (calcein-AM, Invitrogen C3099) to be retained within live cells, including an intense uniform green fluorescence and ethidium homodimer (EthD-1) to bind the nuclei of damaged cells. Following treatments as noted above, PRNCs were incubated with 2 μM calcein-AM and 4 μM EthD-1 for 45 min at RT in dark. After washing once with phosphate buffer saline, PBS, (Sigma-Aldrich P-3813), the green fluorescence of the live cells was measured by the Gemini EX fluorescence plate reader (Molecular Device), excitation at 490 nm and emission at 520 nm. In addition, trypan blue (Gibco, 15250, 0.4%) exclusion method was conducted and mean viable cell counts were calculated in 4 randomly selected areas (1 mm², n = 10) to reveal the cell viability. Briefly, within 5 min after adding trypan blue, we digitally captured under microscope (200×) 10 pictures (approximately 100 cells/picture) for each condition, then randomly selected 5 pictures, and counted the number of cells for each individual treatment condition. Normalized cell viability was calculated from the following equation: viable cells (%) = [1.00 - (Number of blue cells / Number of total cells)] × 100. To precisely calibrate the cell viability, the values were standardized from fluorescence intensity and trypan blue data [24].

Measurement of mitochondrial activity

Following PRNC treatments as detailed above, reduction of 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyltetrazolium bromide (MTT) (Roche 11465007001) by mitochondrial dehydrogenases [24] and WST-1 (4-[3(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate; 05015944001, Roche) by mitochondrial succinate tetrazolium reductase were used as a measure of mitochondrial activity as previously described [26], respectively. Briefly, the cells were treated with 0.5 mg/ml MTT solution at 37°C for 4 h, and subsequently incubated with lysis buffer for 14 h in the incubator in a humidified atmosphere at 37°C and 5% CO₂. The optical density of solubilized purple formazan was measured at 570 nm on a Synergy HT plate reader (Bio-Tex). The cells were treated with 10 μl WST-1 solution at 37°C for 4 h, and subsequently, and measured absorbance at 450 nm.

Western blot analysis

After PRNC treatments as above, the cells were treated with CellLytic MT mammalian lysis reagent (Sigma-Aldrich, C3228) with protease inhibitor cocktail (Sigma-Aldrich, I3786). The lysate was centrifuged at 3,000 g and 4°C for 15 min, and the supernatant was stored at -80°C until used. Protein samples (5~30

µg/lane) were run on 12% Tris-Glycine SDS-PAGE gel [27] and then transferred onto a nitrocellulose membrane (Bio-Rad, 162-0112) at 30 V, 4°C for 14 h. The nitrocellulose membranes were treated with PBST and 5% non-fat milk (Bio-Rad, 170-6404) for 45 min at RT. Membranes were then incubated with the primary antibodies, anti-glutamate decarboxylase67 (GAD67) mouse monoclonal antibody (Abcam, ab26116, 1/1,000), anti-GAD65 mouse monoclonal antibody (Abcam, ab26113, 1/1,000), anti-glutamate dehydrogenase (GLUD1/2) goat polyclonal antibody (Abcam, ab99470, 1/1,000), anti-glutamine synthetase (GS) rabbit polyclonal antibody (Abcam, ab49873, 1/5,000), anti-glutaminase (GLS1/2) rabbit monoclonal antibody (Abcam, ab156876, 1/1,000), anti-HMGB1 rabbit polyclonal antibody (Abcam, ab18256, 1/5,000), and anti-glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) [6C5] mouse antibody (Abcam, ab8245, 1/5,000) at 4°C for 14 h. After washing with PBST, the nitrocellulose membrane was incubated with donkey anti-mouse IRDye800®CW secondary antibody (LI-COR 926-32212, 1/5,000) or donkey anti-rabbit IRDye800®CW secondary antibody (LI-COR 926-32213, 1/5,000) for 90 min at RT in dark. Immunoreactive detection using near-infrared fluorescence was performed according to protocol of Odyssey® Infrared Imaging System (LI-COR®). The relative band intensity was quantified by using NIH image 1.59 software to determine expression level.

Immunoprecipitation (IP) assays

IP assays were performed according to the manufacturer's protocol supplied for Co-IP Kit (Thermo Scientific, 26149). PRNC whole cell lysate (500 µg/100 µl PBS containing with protease inhibitors (Sigma-Aldrich, I3786)) was interacted with anti-HMGB1 rabbit polyclonal antibody (Abcam, ab18256) with beads at 4°C for 16 h. Following antibody-beads complexes were treated with elution buffer, immune complexes were recovered by heating the samples at 95°C for 5 min in Laemmli SDS lysis buffer (BioLand Scientific LLC, SAB02-01). Immunoblotting (IB) analysis performed with anti-acetyl-Lysine monoclonal antibody (Millipore, 05-515, 1/500), anti-phospho-Ser/Thr/Tyr mouse monoclonal antibody (Antibody, ABIN300088, 1/50), anti-ubiquitin mouse monoclonal antibody (Abcam, ab7254, 1/500), and anti-carbonyl rabbit polyclonal antibody (Cell Biolab Inc., STA-308, 1/1,000).

Measurement of glutamate- and major histocompatibility complex II (MHC II)-concentration

Conditioned samples from each experimental condition were processed for measurements of glutamate concentration and MHC II concentration following the manufacturer's protocols for glutamate ELISA kit (Novus, KA1909) and the rat MHC II class ELISA kit (ABIN, 812198, CD74), respectively.

Data analysis

Data were evaluated using one-way analysis of variance (ANOVA) followed by post hoc compromised t-tests. Statistical significance was preset at $p < 0.05$. Data are represented as means $\pm$ SD from 3~6 replicates of each treatment condition.

Results

Translocation of HMGB1

We have shown previously that PRNCs viability and mitochondrial activity were significantly decreased in a dose-dependent manner with KA administration, which is an excitotoxic reagent commonly used to induce epilepsy [23]. Here, we observed that following incubation of PRNCs in 10 µM KA for 3 days at 37°C, HMGB1 translocated from nuclei to cytosol (Fig. 1A, yellow arrows, and Fig. 1B) and to extracellular space (Fig. 1C), although the majority of HMGB1 protein remained in the nuclear region (cytosol HMGB1; $19.5 \pm 10.2\%$, $p < 0.05$). These results were consistent with previous report [28], and suggested that HMGB1 released from nucleus is capable of inducing signal transduction. Moreover, using MAP2 to label neurons, and non-MAP2 positive stains to mark non-neuronal cells (i.e., astrocytes), HMGB1 translocation from the nucleus to the cytosol was comparable between neurons and astrocytes in the present mixed cell culture paradigm.

Fig. 1. KA-induced HMGB1 translocation. (A) HMGB1 translocated from the nucleus to the cytosol fraction (yellow arrows) in response to KA (bottom panel comparison with top panel). PRNCs were cultured with 10 μ M KA (KA) or in the absence of KA (CONT) for 3 days 37°C and stained for neuron (MAP2, red), nucleus (blue, Hoechst), and HMGB1 (green). Scale bars = 5 μ m. (B) Quantification of HMGB1 translocation into cytosol fraction. (C) Measurement of extracellular HMGB1 concentration incubated with 10 μ M KA (KA), 10 μ M KA and 10 μ g/ml anti HMGB1 antibody (KA + Antibody), or with the absence reagent (CONT) for 3 days 37°C. * $p < 0.05$.

Fig. 2. KA induced-HMGB1 post translation modification. After cultured PRNCs were incubated with 10 μ M KA (KA) or in the absence of KA (CONT) for 3 days at 37°C, IP analysis (right panel) using anti-HMGB1 antibody directed at acetylation, phosphorylation, ubiquitination, but not carbonylation. Left panel shows the Western blot analysis of HMGB1. Each rectangles' top and bottom line indicated the molecular weight of 40 kDa and 20kDa, respectively. Experiments were independently conducted for 3 times.

Protein modification of HMGB1

Post-translational modification has been demonstrated to exert a pivotal role for the translocation of HMGB1 [6, 28-32], therefore, we sought to determine whether KA treatment in PRNCs triggered HMGB1 post-translational modification. Immunoprecipitation (IP) analysis demonstrated that HMGB1 was acetylated (Fig. 2 right panel, black arrow), phosphorylated (Fig. 2 right panel, black arrow), and, interestingly, ubiquitinated (Fig. 2

Fig. 3. Effects of KA and HMGB1 on cell viability and relative mitochondrial activity. After cultured PRNCs were incubated with 10 μ M KA (KA), 10 μ M KA and 1 μ g/ml HMGB1 (KA + HMGB1), 1 μ g/ml HMGB1 (HMGB1), 1 μ g/ml HMGB1 and 10 μ g/ml anti HMGB1 antibody (HMGB1 + Antibody), or in the absence of reagent (CONT) for 3 days at 37°C, cell viability and mitochondrial activity were measured by using calcein assay (A), MTT assay (B), and WST-1 assay (C), respectively. *a* vs *a* $p < 0.01$, *b* vs *b* $p < 0.001$, *c* vs *c* $p < 0.001$, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Experiments were independently conducted for 4 or 5 times.

right panel, red arrow), and showed two bands which suggested multiple steps of protein modification [33]. These observations suggested that post-translational modifications after KA treatment caused HMGB1 to dissociate from chromatin DNA, and promoted the release of HMGB1 into the extracellular milieu (Fig. 1C).

Extracellular HMGB1 alters cell viability and mitochondrial activity

We evaluated whether extracellular HMGB1 affected cell viability and mitochondrial activity of PRNCs. Following incubation of PRNCs in 1 μ g/ml HMGB1 for 3 days at 37°C, cell viability and mitochondrial activity were significantly decreased (p 's < 0.001 ; Fig. 3). After co-treatment with 1 μ g/ml HMGB1 and 10 μ g/ml anti-HMGB1 antibody (Abcam, ab79823), both cell viability ($F_{2,16} = 58.04$, $p < 0.0001$) and MTT activity ($F_{2,12} = 53.5$, $p < 0.0001$), and WST-1 activity ($F_{2,12} = 64.82$, $p < 0.0001$) were significantly recovered compared with HMGB1 alone. Interestingly, mitochondrial activity was more vulnerable to HMGB1 than KA (Fig. 3B, $p < 0.01$ and Fig. 3C, $p < 0.05$).

Extracellular HMGB1 downregulates expression levels of glutamate metabolism-associated enzymes

Initiation of epilepsy is generally viewed as resulting from an imbalanced excitatory/inhibitory equilibrium, where an excitatory transmitter is glutamate and an inhibitory transmitter is γ -aminobutyric acid (GABA). We examined whether HMGB1 altered the expression level of glutamate metabolism-associated enzymes in epilepsy-related hyperexcitability (Fig. 4A). Following incubation of PRNCs with 1 μ g/ml HMGB1 for 3 days at 37°C, the expression levels of GAD67 and GLUD1/2, but not GAD65, were significantly decreased (p 's < 0.001 ; Fig. 4B and C). Accordingly, HMGB1-exposed PRNCs shifted the

Fig. 4. KA and HMGB1 alter expression of glutamate metabolism-associated enzymes. PRNCs were incubated with 10 μ M KA (KA), 1 μ g/ml HMGB1 (HMGB1), or in the absence of reagent (CONT) for 3 days at 37°C. (A) Schematic diagram details glutamate metabolism-associated enzymes. (B) Western blotting analysis of glutamate metabolism-associated enzyme expression in PRNCs. (C) Relative quantification of protein expression levels. Experiments were independently conducted for 3 or 4 times. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

expression level of glutamate/GABA-synthesized enzymes, suggesting that HMGB1 signaling contributes to initiation of epileptic seizures.

Extracellular HMGB1 increases the intercellular glutamate concentration and MHCII expression levels

We examined whether extracellular HMGB1 increased intracellular/extracellular glutamate concentrations (Fig. 5A and B). Following incubation of PRNCs with 1 μ g/ml HMGB1 for 3 days at 37°C, intracellular glutamate concentration was significantly increased (Fig. 5A, $p < 0.05$). However, we could not detect any significant changes in extracellular glutamate concentration (Fig. 5B, $p = 0.748$). Furthermore, we evaluated whether extracellular HMGB1 affected expression of MHC II (CD74 or OX6; invariant chain), which is a neuroinflammation marker [34, 35] sensitive to both exogenous and endogenous antigenic proteins [34]. We observed that MHC II expression levels were significantly increased (Fig. 5C, $p < 0.05$), indicating that exogenous HMGB1 accompanied the initiation of neural cell death processes.

Discussion

The present study demonstrated that KA treatment of PRNCs induced the translocation of HMGB1 from nucleus to cytosol, resulting in post-translational modifications. The KA-induced increment in extracellular HMGB1 was neurotoxic, decreasing PRNC cell viability and mitochondrial activity, downregulating GAD67 and GLUD1/2 expression, and increased intracellular glutamate and MHC II concentrations. These data suggest that HMGB1 participates in seizure initiation, which is a key pathophysiological feature of stage epilepsy-related hyperexcitability.

Pathological analyses of patient brain tissues with refractory epilepsy indicate rampant neuroinflammatory processes. HMGB1, which acts like an inflammatory cytokine, is increased in epileptogenic human brain tissue and in chronic mice [12]. The considerable influence

Fig. 5. Effects of KA and HMGB1 on intracellular/extracellular glutamate concentration and MHC II expression levels. PRNCs were incubated with 10 μ M KA (KA), 1 μ g/ml HMGB1 (HMGB1), or in the absence of reagent (CONT) for 3 days at 37°C. (A) Intracellular glutamate concentration. (B) Extracellular glutamate concentration. (C) Expression levels of MHC II in PRNCs. Experiments were independently conducted for 3~6 times. *a* vs *a* p < 0.05, * p < 0.05, and ** p < 0.01.

of neuroinflammation in epilepsy implicates the involvement of excitable elements in brain disorder, in particular energy metabolism requiring metabolic control [36]. Along this line of investigations, KA excitotoxic animal model has been widely accepted as a model of epilepsy. Commonly used model of mesial temporal lobe epilepsy include acute systemic injection of KA [12, 20, 22]. We recently reported the utility of KA in resembling many of the pathological features of early stage epilepsy-related hyperexcitability in cultured PRNCs [16]. P-glycoprotein (P-gp), a membrane-bound transporter [37], is associated with inflammatory process in the epileptic brain [38]. At 24 h after KA injection into the mouse hippocampus, nuclear-to-cytoplasmic translocation of HMGB1 ensues and closely accompanies the upregulated expression of P-gp in the post-seizure brain [39]. These data collectively indicate that KA-induced HMGB1 translocation is associated with a considerable neuroinflammatory response, which we showed here was characterized by MHC II upregulation.

Using this *in vitro* KA paradigm in the present study, we demonstrated HMGB1 translocation from nuclear to cytosol, and extracellular space (Fig. 1). The observation that HMGB1 was released from the damaged cells exposed to KA excitotoxicity offers novel post-translational modifications in the pathology of epilepsy-related hyperexcitability. KA induces Golgi-ER complex fragmentation/dispersal and shifts the proteolysis in PRNCs [23]. To date, KA interacting with HMGB1 remains unexplored, however, previous studies provide some clues on the possible participation of HMGB1 in cell function. HMGB1 is independently secreted through a Golgi-ER secretory pathway due to the lack of signal peptide [40]. It is transported from the nucleus to the cytoplasm and then into the lysosomes and ultimately released in to the extracellular space through exocytosis. HMGB1's accumulation in secretory lysosome suggests the involvement of Ca^{2+} regulated secretion [41]. Acetylation [42] and

phosphorylation [43] of HMGB1 regulate its intracellular localization and extracellular secretion, reflecting its ability to function as an inflammatory mediator. In this study, HMGB1 was acetylated and phosphorylated by KA-administration (Fig. 2, right panel). These findings are consistent with previous studies demonstrating that inflammation- and cellular stress-mediated acetylation of HMGB1 prevents its nuclear reentry and leads to the accumulation of HMGB1 in cytoplasm [44]. Interestingly, IP analysis (Fig. 2 right panel, red arrow) revealed that KA-induced HMGB1 mobility was approximately 8 kDa shorter than Western blot analysis (Fig. 2 left panel, blue arrow) and indicated that HMGB1 is mono ubiquitinated. This mono ubiquitination regulates the target protein's cellular functions, such as membrane trafficking [45, 46] and exocytosis [47]. These results provide novel evidence that the shuttling of HMGB1 between the nucleus and extracellular space may be tightly regulated in response to an epileptogenic signal. A limitation of the study is the use of pan-modification markers to demonstrate acetylation, phosphorylation and ubiquitination of HMGB1. The observed positive bands may represent modified proteins that interact with HMGB1, based on the similarity of size and patterns of all three targets characterized as two bands around 35-40kd, with a strong and a weak upper and lower band, respectively. Future studies using HMGB1 specific antibodies should reveal discrete levels of acetylated, phosphorylated, and ubiquitinated HMGB1.

Administering two different types of chemoconvulsants KA and bicuculline (a GABA_A antagonist) resulted in acute increases in HMGB1 at mouse hippocampus [12]. Constitutive exposure of HMGB1 is associated with chronic epilepsy in rat model and in human epileptic tissues [48]. Here, we showed KA and HMGB1 afforded comparable neurotoxic effects in decreasing PRNC cell viability (Fig. 3A). Our results (Fig. 3) concur with a previous report that the combined injection of KA and HMGB1 into mouse hippocampus significantly increases the seizure area compared with the effect produced by KA alone [12]. Of note, treatment with HMGB1 alone in the present study significantly dampened PRNC mitochondrial activity, comparable to the effect of KA alone (Fig. 3B and C). Mitochondria are the main producers of ATP needed for neuronal electrical activities and synaptic transmission. In the progression of epilepsy, mitochondrial dysfunction closely coexists with epileptogenesis and chronic epilepsy [49]. Altogether, these results suggest that a deficient ATP maintenance following HMGB1 exposure corresponds to the early stages of epilepsy-related hyperexcitability. Because the present study employed the mixed culture system, the observed cell viability and mitochondrial activity most likely reflected a mixed response of both cultured neurons and astrocytes. In order to reveal the specific proliferative and apoptotic activity of astrocytes separate from the neurons, additional experiments are using pure cell culture systems. Notwithstanding, that KA induces neuronal death and astrogliosis *in vivo* might have been reproduced here by our mixed culture system. In view of astrocytes being activated by HMGB1 and acquiring a reactive phenotype, which results in increased production of various chemokines and cytokines, this cell death-mediated effect alone might result in mortality of the cultured cells.

Extracellular glutamate is taken up by glutamate transporters and rapidly converted to the non-excitotoxic amino acid glutamine by GS in a process requiring ATP (Fig. 4A). In the brain, GS functions as an inhibitor of glutamate-dependent excitotoxicity [50]. Intracellular excessive glutamate is converted into GABA and/or α -ketoglutarate by GAD65/67 and GLUD1/2, respectively (Fig. 4A). KA and HMGB1 significantly decreased the expression levels of GAD67 and GLUD1/2, but both reagents did not produce any pronounced effect on GAD65 (Fig. 4). GAD67 tightly binds the coenzyme pyridoxal 5-phosphate (PLP) and is constitutively active (holoenzyme form), robustly producing GABA in the brain [51, 52]. In contrast, GAD65 depends on the interconversion between an active PLP-bound holoenzyme and an inactive apoenzyme [53], requiring the activation of extra GABA synthesis. Moreover, GAD67 is more ubiquitously distributed within neurons and associated with the synthesis of GABA released. PLP is a cofactor for GAD67 and its deficiency can induce neonatal epileptic encephalopathy [54]. Because GAD67 acts as the major catalyst of GABA production [51, 52], a deficit in GAD67-mediated GABA synthesis is likely to reduce the GABAergic inhibition

in the brain. It is conceivable that under KA treatment, a synergistic effect of decreased GS expression (Fig. 4) and suppressed mitochondrial activity (Fig. 3B) in PRNCs may amplify neural cell death processes. This hypothesis is supported by the observation that KA and HMGB1 significantly increased intracellular glutamate concentration and upregulated MHC II expression (Fig. 5).

Our data revealed that KA induced an intracellular HMGB1 translocation that was exquisitely regulated by post-translation modifications, with extracellular HMGB1 shifting the glutamine/GABA equilibrium in PRNCs, capturing the early stages of epilepsy-related hyperexcitability initiation. We recognize the need to determine the downstream pathways (i.e. acetylation, phosphorylation, and ubiquitination) and to complement with *in vivo* studies this observed KA-HMGB1 *in vitro* interaction in order to strengthen the clinical relevance of the findings. Because epilepsy, is a network disease, there is limited direct translational application of the present cell culture paradigm, which represents an artificial system with a specific number of neural subtypes. Nonetheless, the present results advance the concept of HMGB1 post-translational modifications [28, 55], in our understanding of disease pathology and developing treatments for epilepsy-related hyperexcitability.

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Disclosure Statement

The authors state that they have no conflict of interest.

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