IJISET - International Journal of Innovative Science, Engineering & Technology, Vol. 2 Issue 8, August 2015.
www.ijiset.com
ISSN 2348 – 7968

Alteration of Brain expression of prolyl endopeptidase in animal model of minimal hepatic encephalopathy.

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Abstract
Minimal hepatic encephalopathy (MHE) produced mild motor and cognitive disorders and predisposes to suffer hepatic encephalopathy (HE), the most severe form of the disease. Currently, hyperammonemia (HA) and neuroinflammation are considered synergic factors involved in the pathogenesis of MHE and HE. The serine peptidase prolyl endopeptidase (PREP) cleaves short peptides (<30 amino acids) at the C-side of a proline residue. It has been proposes that PREP may play a role in the inflammatory response through peptide modulation. Objectives: In this paper we showed our results in relation to the expression of PREP in a model of MHE produces by induction of chronic moderate HA. Methods: Brain samples were collected from HA rats and PREP expression was determined by immunohistochemistry analysis and immunoblotting assessment in the neocortex, hippocampus, striatum and cerebellum. Results: The results indicated that HA produces PREP expression alterations in most of the areas analyzed. After 8 weeks of HA the expression of PREP was increased in the motor cortex, CA1 region of the hippocampus, the caudate putamen of the striatum and in the cerebellum. Conclusions; these results may indicate that prep increase may have a role in alterations seen in hyperammonemia without liver impairment.

Keywords: *Minimal hepatic encephalopathy*, *ammonium*

1. Introduction

Hepatic encephalopathy (HE) is a complex neurological disorder produces by a liver failure. Depending of the type of liver lesion, acute or chronic, neurological alterations may covers an amply spectrum range from subtle alterations of the cognition and motor abilities, impairments in sleep-wake cycle, attention, mood and psychomotor alterations to coma and death. Most chronic liver diseases patients present minimal hepatic encephalopathy (MHE), e.g. 30-50 % of patients with liver cirrhosis suffer MHE [1] . MHE is characterized by mild cognitive impairment, attention deficits, psychomotor slowing and impaired visuomotor and bimanual coordination[1] .The pathogenesis of HE and MHE involves a multifactorial process triggered by a liver failure. Traditionally, the increased ammonium level in the brain has been recognized as the key factor in the HE development, a process named hyperammonemia (HA). However, in the last years has been recognized the synergic action of neuroinflammation and HA as main factors involves in the development of MHE and HE [2] . Recently, our group demonstrated that HA without liver lesion produces neuroinflammation, especially in the cerebellum [3]. The molecular mechanism underlie the neuroinflammation respond to ammonium remains unclear but the reduction of neuroinflammation has been related with the recovery of cognitive and motor deficits in HE and HA animal models. For example the administration of the MAPK-p38 inhibitor reduced the neuroinflammation and restored the cognitive deficits in a HE animal model [4] or the use of NAISD has been documented restores the microglia activation in HA animal model [5].
Recently, has been described that the serine peptidase prolyl endopeptidase (PREP or POP, PEP, PE) may play a role in the inflammatory response through peptide modulation[6], [7] PREP cleaves short peptides (<30 amino acids) at the C-side of a proline residue [8]. The involvement of PREP has been detailed in different inflammatory diseases [9]–[14] . Furthermore, activity alterations of PREP have been associated with memory and learning deficits [15] and the administration of PREP inhibitors restored the memory impairments in different brain pathologies in rodents and primates [16]. Moreover, different authors has described a possible relation of PREP with different neurological diseases, such as Alzheimer or Parkinson [8]. The brain expression of PREP has been well characterized in different brain areas, such as

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hippocampus, striatum, neocortex and cerebellum. In humans and rodents PREP expression is localized in specific brain areas and cell types [17]. In the healthy brain the positive expression of PREP is localized principally in specific neurons types but not in glia cells [10], [12]. However, under pathological conditions, such as neuroinflammation, has been described that PREP is expressed in glia cells and the increased expression is related with neuronal neurotoxicity [18], [19]. PREP released from activated microglia may contribute to the neurotoxic actions of these cell and the neurotoxicity of this secretion can be reduced by the use of PREP inhibitors [10]. Inhibitors of PREP have been proposed as potential therapeutic drugs to reduced neuroinflammation and recover cognitive functions in different neuropathology diseases [12], [20], [21]. In this paper we showed our results in relation to the expression of PREP in a model of HA without liver failure that mimic MHE. Our model of HA produces neuroninflammation and subtle cognitive and motor impairments that reproduces some of the behavior hallmarks of MHE [3]**.** This is the first analysis of brain PREP expression in a model of hepatic encephalopathy without liver damage.

2. Materials and methods

2.1 Animal model of hyperammonemia

Male Wistar rats (175-200 g) were made hyperammonemic by feeding them an ammonium-containing diet for 8 weeks as previously described [22]

2.2 Determination of blood Ammonia

Determination of blood Ammonia was measured with The PocketChem BA blood ammonia analyzer (Menarini Diagnostics, Florence, Italy) for whole blood ammonia concentration. We collect a 20µL of blood sample with the attached pipette and drop it on the reagent strip. The Ammonia Test Kit II adopts dry chemistry based on a micro-diffusion method. The degree of color development after 3 min of incubation is measured calorimetrically and is proportional to the ammonia concentration in the sample.

2.3 Immunohistochemistry

Rats were perfused transcardially under deep anesthesia (pentobarbital, 100 mg/kg, i.p.) with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Coronal 30 µm sections were cut on a cryostat (Leica VT1000-S) and were stored at 4ºC in PB with 0.1% azide until further processing. Free-floating sections were washed, endogenous peroxidase activity was quenched with 3% H_2O_2 for 15 min, and sequential incubations with blocking serum and primary antibody (1:300, affinity purified chicken IgY,[17], [19], [23] was added and incubated overnight at 4 °C. To label PREP + cells we sequentially incubated with donkey anti chiken - horseradish peoroxidase complex (Jackson inmuno research laboratories inc). DAB-H_2O_2 substrate was used to label antigenic sites and Niquel sulphate was added to improve the contrast label. The stained sections were mounted on slides, dehydrated and coverslipped.

2.3 Western blotting

Samples from the brain were diluted 1:1 with loading buffer (100 mm Tris/HCl, pH 6.8, 70% glycerol, 2% SDS, 0.005% bromophenol blue, 10 mm mercaptoethanol) and separated on 10% polyacrylamide/bis-acrylamide Tris/HCl discontinuous gels. Gels were transferred to nitrocellulose for blotting. Western blot was performed under standard conditions using affinity purified chicken IgY, [17], [19], [23] diluted 1:500 with 0.5 m NaCl, 20 mm Tris–HCl pH 7.5, 5% skim milk and 0.05% Tween 20), and anti-chicken horseradish peroxidase complex (Santa Cruz Biotechnology Inc., Dallas, TX, USA) diluted 1:3000. Protein visualization was performed using a chemoluminescent substrate kit (Pierce, Rockford, IL, USA), following the manufacturer's instructions.

2.4 Quantification analysis

Tissues were observed in a vertical microsocope Leica DMR 6000 microscope and images were captured with a Leica DFC 480 camera. Low-magnification images presented were photographed for cortex, striatum, hippocampus and cerebellum and exported for cell counting within Image J 1.46 (NIH) or optical density analysis. The optical density assessment was carried out using a free-hand tool of Quantity One-software (version 4.6.9, Bio-Rad). Staining quantifications of positive PREP cells were performed in 3- 4 areas (0.08 mm^2) of 5-6 sections randomly chosen per animal. Western blot images were scanned and analyzed and the optical density (OD) values of protein bands were detected by using a free-hand tool of Quantity One-software (version 4.6.9, Bio-Rad). The result was expressed as a percentage respect to control.

2.7 Statistics

Values are given as mean ± standard deviation. The data were analyzed by Two-tailed t student test. Significance levels were set at alpha = 0.05.

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3. Results

3.1 Ammonium level assessment

The rats fed with the ammonium diet showed a significant increases in the ammonium blood level (54.75 ± 8, 32) in the HA group respect the control values (14, 75 ± 4, 04). T-student analysis indicated a significant difference ($p < 0.005$.)

3.2 PREP expression

a. Western blot

Using immunoblotting we assessed PREP expression in cerebellum (CB), hippocampus (HP), cortex (CTX) and striatum (STR) after 8 weeks of ammonium diet. The results indicated a significant increase in PREP expression in brain areas analyzed, in the CTX (149, 5 ± 19, 33) of the HA group respect the control values (100 ± 12, 76). In the HP (140,6 ± 12,95) respect to its control (100,0 ± 7,824) and In the striatum (135,8 ± 6,989) respect to its control (100,1 ± 11,85) and in cerebellum (138,5 ± 23) Vs (100,0 ± 4,326) . Representative images are represented in **Figure 1**.

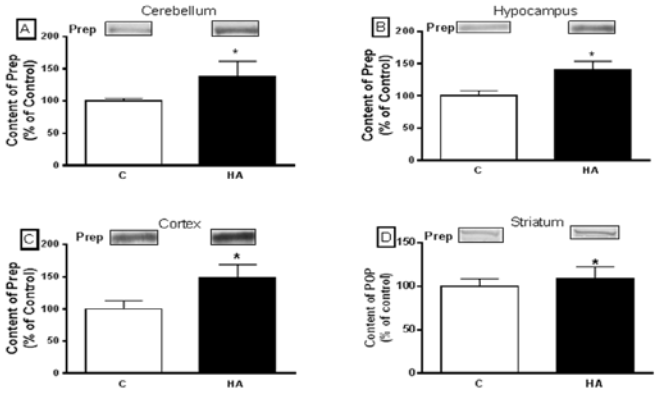

Fig 1: PREP content were analyzed by immunoblotting. Representative immunoblot is shown in (A) for cerebellum, (B) for Hippocampus, (C) for Cortex and (D) Striatum .The intensities of the bands were quantified and expressed as percentage of controls. Values are the mean ± SEM. The differences between groups are indicated by asterisk.* $p<0.05$.

b. Inmunohistochemistry analysis

Analysis of PREP inmunoreactivity in specific brain areas and cell populations requires higher resolution techniques than western blot. We analyze high PREP reactivity brain areas, including motor cortex, caudate putamen and CA1 regions by microscopy analysis. In the CA1 region of the hippocampus we assessed PREP expression by optical density analysis. The result indicates that PREP expression was increased in the CA1 region of the HA group (247, 6 ± 43, 17) group almost 2.5 fold respect the control level (100 ± 14,12). The quantitative analysis of positive PREP cells in the motor cortex indicate a significant difference in the HA group (167,0± 24,59) respect to the control group (100±3,391). The analysis of PREP inmunoreactivity in the caudate putamen region indicated an increased near of 3 fold the control values. Statistical analyses indicated significant difference between the experimental groups ($p<0.003$), HA group showed an increases (277,6± 5,832) versus control values (100±8,620) and the cerebellum indicate an increase of (151,2 ± 7,251) respect to its control (100,0 ± 6,868) . The microscopy analyses indicated PREP alterations in three regions with high PREP inmunoreactivity, the CA1 region of the hippocampus, the primary motor cortex and the caudate putamen of the striatum see *Figure 2*.

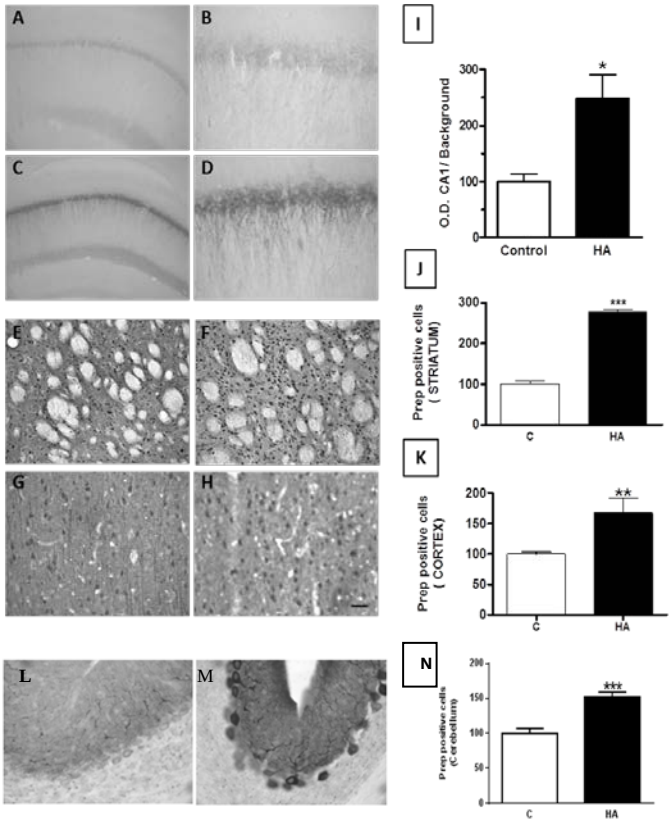

Figure 2. The figure showed stained positif Prep cells per mm2 by Immunohistochemistry and quantification of Diferent brain areas of rats .Sections from control and hyperammonemic rats were used to analyze the expression Prolyl Endopeptidase . Images from coronal sections of CA1 region of Hippocampus control group (A, B) and hyperammonemic group (C,D) also images of striatum Control (E) and Hyperamonemia (F) , cerebellum Control (G) and Hyperamonemia (H) , motor cortex Control (L) and Hyperamonemia (M) are represented. Chronic hyperammonemia increase the amount of Prep in all brain areas of HA rats. C: Control, HA: Hyperamonemique. Values are the mean ±standard error. Values significantly different from control rats are indicated by asterisks. *P < 0 .05, **p<0.01. Scale bar 200 µm in A, C and 50 µm in B and D.

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3 Discussion

To our Knowledge this is the first study to examine the regional distribution and localization of PREP protein by western Blot and immunohistochemistry successively in the brain of hyperamonemic rats. We confirmed that prep is expressed in Hippocampus, Cortex, striatum and cerebellum of control rats and we showed that HA Rats displayed an increase in prep expression protein levels .

Our results show that PREP was present in the cortex, striatum, hippocampus and cerebellum, as has been described in several studies [17], [18] . Those areas participate in cognitive and motor functions, such as the [24]. Furthermore PREP is present in glutamatergic, gabaergic, and cholinergic systems [25] . Moreover hydrolysis of neuropeptides is the main function of PREP in the brain[18] and changes in these neuropeptide levels have been reported in several neurodegenerative diseases [26], [27] .

In the present study, we have showed that PREP is high expressed in those brain areas of ha rats compared to it control. We used immunohistochemistry to confirm the results and we find that PREP is hight expressed in specific cells,this distribution confirms the role of prep in degrading some neuropetides in that brain area . On the other hand it has been recognized for a long time that hyperammonemia is a main contributor to the alterations in cerebral function and neurotransmission in HE and MHE [28], [29].

In previews study was shoowen that the glutamatergic neurotransmission is altered in primary motor cortex [30] and In cortex prep is colocalised with glutamergique neurons abundantly[18], [31] . Furthermore ,PREP expression was founded icreased in the striatum after 8 weeks of diet also the cellular expression of prep in The caudate putamen of the striatum determined an increase of the level of prep expression in HA rats, It was well documented in striatum a motor impairment respond in HE encephalopathy models[32] .additionaly PREP have a role in the in modifying the GABAergic signaling via the substance P that occurs in Parkinson's disease[19] .In cerebellum prep was colocalized in cellular purking with gabaergique [19] studies in our laboratory have shown that hyperammonemia Increases GABAergic Tone in the cerebellum impairs the function of the glutamate nitric oxide pathway associated with NMDA receptors in cerebellum AND The alteration of this molecular pathway has been associated with conditional learning impairment in an HA model [30] . That could explain a role of prep in the motor alteration seen in HA.

The hippocampus after 8 weeks of ammonium diet show a hight expession in the PREP in the CA1 layer in HA rats respect to control rats , In previews report prep was highly colocalized with IP3R1 in hippocampal CA1 pyramidal cells.[18]In these cells, IP3 has been associated with long-term potentiation (LTP), an important phenomenon in memory and learning[33], [34] .Some study showed that Long terme potentiation is impaired in hippocampal slices from rats with chronic hyperammonemia[35] ,this suggests that Prep in hyperamonemic rats may have a role in alterations seen in memory function in HA .

On the other hand prep has a role in neuroinflammation and our animal model produces neuroinflammation [2]. Indeed The ammonium increased in the blood and brain produces microglia and astrocytes . furthermore the inflamatory markers are increased such as il-8 and il-6 in Hyperammonemia and prep was coexpressed with some inflammatory factor. This suggest that prep have a role in inflammation in hyperammonemia .

Acknowledgments

This work was supported by the European Project N€uromed (FP7-REGPOT-245807**).**

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