Biomedicine & Pharmacotherapy 88 (2017) 762–769

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Original article

Neuroprotective effect of chrysin on hyperammonemia mediated neuroinflammatory responses and altered expression of astrocytic protein in the hippocampus

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ARTICLE INFO

Article history:
Received 25 October 2016
Received in revised form 12 January 2017
Accepted 12 January 2017

Keywords:
Chrysin
NH_4Cl
Hyperammonemia
Brain edema
Neuroinflammation

ABSTRACT

Neuroinflammation is an innate immune response in the central nervous system (CNS) against metabolic and pathogenic toxic wastes. The main hypothesis implies that a state of hyperammonemia which is accountable for both direct and indirect modification in ammonia metabolism with an elevated production of inflammatory cytokines. This study was constructed to explore the modulating effect of chrysin on rudimentary pathophysiologic mechanisms of ammonium chloride (NH_4Cl) mediated neuroinflammation in the experimental hyperammonemic rats. NH_4Cl was injected intraperitonally *(i.p)* in male albino wistar rats for a time period of thrice a week for eight consecutive weeks. Initially, the levels of brain ammonia and water content were assessed. Immunohistochemical, RT-PCR and western blotting analysis revealed that the expression of glutamine synthetase (GS) activity and glial fibrillar acidic protein (GFAP) were down-regulated, whereas the expression of TNF-α, IL-1β, IL-6, p65 NF-κB, iNOS and COX-2 were up-regulated in brain tissue of hyperammonemic rats. Oral supplementation of chrysin (100 mg/kg b.w) to hyperammonemic rats considerably restored the levels of brain ammonia, water content, and the expressions of GS, GFAP, TNF-α, IL-1β, IL-6, p65 NF-κB, iNOS and COX-2. Our findings provided substantial evidence that the chrysin synergistically attenuating the neuroinflammatory mechanism by repressing the expression of proinflammatory cytokines and up-regulating the astrocytic protein expressions via ammonia-reducing strategies. This data suggests that chrysin effectively acts as a therapeutic agent to treat hyperammonemia mediated neuroinflammation.

1. Introduction

Hyperammonemia is a major contributor to neurological modifications found in hepatic encephalopathy (HE) related with acute and chronic liver disease [1]. In liver failure, the results turn into the impaired detoxification of ammonia that increases blood ammonia, also reaches the brain and alter its function. Subsequently, brain ammonia increases in the range of 3–5 mM [2]. Ammonia is the best characterized CNS toxin, which trigger a variety of neurological complications. Elevated concentration of brain ammonia leads to physiological disturbances, biochemical and morphological alterations [3]. As a result, it alters the astrocytic protein expression including GFAP and GS. Ammonia is believed to the major factor for astrocytes swelling implicated in hyperammonemia induced brain edema. Astrocytes predominantly detoxify ammonia by the formation of glutamine from amidation of glutamate by glutamine synthetase. Moreover, elevated levels of brain ammonia have been considered as an important etiological factor of brain edema, which induces neuroinflammation [4,5].

Hyperammonemia plays important role in neuroinflammation and neurological alterations in HE [6]. A major characteristic feature of neuroinflammation is glial cell activation including microglia and astrocytes. These activated microglia release cytokines and chemokines, which eventually cause neuronal

Abbreviations: GS, Glutamine synthetase; GFAP, Glial fibrillar acidic protein; TNF-α, Tumor necrosis factor alpha; IL-1β, Interleukin-1beta; IL-6, Interleukin-6; p65 NF-κB, Nuclear factor-kappa B; iNOS, inducible nitric oxide synthase; CNS, central nervous system; NH_4Cl, ammonium chloride; HE, hepatic encephalopathy; NMDA, *N*-methyl *D*-aspartic acid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ALF, cute liver failure; GABA, γ-amino butyric acid; H&E, Hematoxylin and Eosin; ROS, reactive oxygen species; CPCSEA, Committee for the purpose of normal and supervision of experiments on animals; RT-PCR, Reverse transcriptase-polymerase chain reaction.

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http://dx.doi.org/10.1016/j.biopha.2017.01.081
0753-3322/

damage and death [7]. Neuroinflammation mediates the alterations of cognitive and motor functions found in hyperammonemia [8]. Ammonia intoxication triggers various changes including reductions in GS activity and the redox state, and it also induces the release of pro-inflammatory cytokines [9]. Systemic inflammation, improved the release of circulating pro-inflammatory cytokines including interleukins and tumor necrosis factor alpha (TNF-α) by which it indicates an evidence for the increased expression of these cytokines in brain [10].

A great deal of paying attention continues to be focused on ammonia intoxication (culprit toxin) associated with the pathogenesis of CNS complications [11]. The interest towards flavonoid comes from the results of epidemiological studies, that increased fruit and vegetable consumption is allied with a lower risk of various diseases [12]. One such flavonoid that has obtained substantial attention is chrysin (5,7-dihydroxyflavone), a hydroxylated flavone found in various plant extracts, including *Passiflora caerulea* (blue passion flower), honey, pine wood and propolis, have great economic value and medicinal impact with minimum side effects. Currently, chrysin has been exhibited to have an inestimable number of manifold biological actions, such as anticancer [13], anti-inflammatory via inhibiting the expressions of pro-inflammatory cytokine [14], also it has been reported that chrysin suppresses the nuclear factor-κB (NF-κB) and inducible nitric oxide synthase (iNOS) [15], and antihypertensive effects [16]. There were no preexisting published reports with respect to neuroprotective effect of chrysin on NH_4Cl induced hyperammonemia mediated neuroinflammation. The focus of this study was to emphasize the influence of chrysin in opposition to astrocytic swelling and neuroinflammatory responses of hyperammonemic rats.

2. Materials and methods

2.1. Chemicals

Chrysin and NH_4Cl were obtained from Sigma Chemical Company, St. Louis, MO, USA. All other chemicals were of analytical grade and purchased from Bangalore Genei, India.

2.2. Animal model and ethical statement

Male albino Wistar rats at 6–8 weeks of age were acquired from Biogen, Bangalore. In accordance with the ethical norms, this experiment was designed and performed with protocols approved by Animal Ethical Committee, Annamalai University & CPCSEA guidelines, New Delhi, India (Approval no. 1118; dated:16/04/2015).

2.3. Experimental design

The rats were separated into four groups, comprising a minimum of 6 rats in each group (Fig. 1).

2.4. Induction of hyperammonemia

Hyperammonemia was provoked in rats by *i.p* injections of freshly prepared NH_4Cl (100 mg/kg b.w.) solution according to the previously described method [17].

2.5. Chrysin

Chrysin in corn oil was administered orally at 100 mg/kg b.w. thrice a week for eight consecutive weeks. The dose was chosen, based on previous studies in the literature [18].

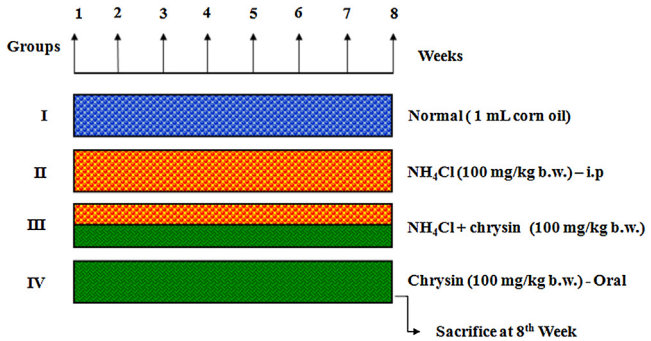

Fig. 1. Experimental design. Group I: Rats received 1 ml of corn oil. Group II: Rats received NH_4Cl by *i.p* (100 mg/kg b.w). Group III: Rats received NH_4Cl + chrysin (100 mg/kg b.w) by oral. Group IV: Rats received chrysin (100 mg/kg b.w) alone. At the end of the experimental period (8th week), all the rats were sacrificed by cervical dislocation. Brain tissues were excised and used for various biochemical estimations and molecular studies.

2.6. Brain ammonia content

Brain ammonia concentration was assessed using a standard kit by the method of [19]. A sample of brain tissue was dissected and homogenized. The homogenates were deproteinized with lysis solution (6% containing trichloroacetic acid) and centrifuged by 12,000 *g* at 4 °C for 10 min. After centrifugation, potassium bicarbonate (2 mol/L, pH = 7) was added to the collected supernatent.

2.7. Brain edema

Brain water content was measured by previously described method [20]. Rat brain tissues were separated and relocate onto pre-weighed glass scales. Wet weight of brain tissue was measured initially and the sample was dried whole night in an oven at 120 °C. Dried brain tissue was subsequently weighed.

2.8. Histological studies

At the end of the experimental period (8^{th} week), rats were fasted overnight and killed by cervical dislocation. Brain tissue of all groups were dissected and washed, then dehydrated in various concentrations of ethanol, cleared in xylene and fixed with paraffin wax. About 5–6 mm in thickness of brain tissue sections was sliced out, deparaffinized and then stained with Hematoxylin and Eosin (H&E) for visualizing under the light microscope (magnification 40×).

2.9. Immunohistochemistry staining analysis

Brain tissue sections were rehydrated by the grade of ethanol to distilled H_2O. The activity of endogenous peroxidase was removed by adding H_2O_2 (3%) for 15 min. Tissue sections were incubated with blocking reagent (BlockTM) at room temperature for 15 min. Sections were incubated with the suitable primary antibody against GS, GFAP, TNF-α, IL-6, iNOS, p65 NF-κB and COX-2 followed by accurately labeling with the secondary antibody with horseradish peroxidase. After the colour intensity was reached, the slides were visualized with hematoxylin staining.

2.10. Transcriptional analysis

Brain tissue was homogenized and RNA was extracted from the tissue using the RNeasy-Mini-Kit and then the extracted RNA was evaluated for purity and concentration in Nanodrop