Role of Bioflavonoid Quercetin on Expression of Urea Cycle Enzymes, Astrocytic and Inflammatory Markers in Hyperammonemic Rats

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Abstract This study evaluates the role of quercetin on the expression of urea cycle enzymes, astrocytic, neuronal and inflammatory markers in hyperammonemic rats. Hyperammonemia (provoked by intraperitoneal injections of ammonium chloride—100 mg/kg b.w for 56 days), showed diminished expression of urea cycle enzymes [carbamyl phosphate synthetase-1 (CPS-1), ornithine transcarbamylase (OTC), argininosuccinate synthetase (ASS) and arginase (ARG)] in liver and decreased expression of neuronal and astrocytic markers—glutamine synthase (GS) and phosphate activated glutaminase (PAG) in brain and increased expression of brain inflammatory markers such as interleukin 6 (IL6), inducible nitric oxide synthase (iNOS) and nuclear transcription factor kappa B (NF- κ B) (by western blot analysis) and exhibited downregulated expression of soluble guanylate cyclase (sGC), glial fibrillary acidic protein (GFAP) in brain and ASS in liver investigated (by RT-PCR). Oral treatment of quercetin (50 mg/kg b.w) to hyperammonemic rats (1) increased the expression of urea cycle enzymes (CPS-1, OTC, ASS and ARG), neuronal and astrocytic markers (GS and PAG) (2) decreased the expression of IL6, iNOS and NF- κ B and (3) upregulated mRNA expression of SGC, GFAP and ASS. Our results specify that quercetin's antihyperammonemic effects could be through its, anti-inflammatory, neuroprotective and hepatoprotective effects.

Keywords Quercetin · Hyperammonaemia · Ammonium chloride · Urea cycle

Introduction

Ammonia is formed in mammals by the catabolism of proteins and other nitrogenous compounds; at elevated levels ammonia is toxic, causing dysfunction of cerebral activity [1–3]. Under normal conditions, a major portion of ammonia is released into portal circulation and is detoxified in liver. In liver diseases (for instance, liver cirrhosis) ammonia is not sufficiently detoxified, causing hyperammonemia and hepatic encephalopathy, neuropsychiatric syndromes and can finally cause coma and death [4].

The molecular mechanisms constituting the hyperammonemia induced dysregulated cerebral function are not completely understood. Hyperammonemia could also be caused by hereditary insufficiency of enzymes linked with urea formation [5, 6], kidney failure [7] and liver diseases [8].

Genetic disorders in the biosynthesis of urea cycle enzymes cause accrual of ammonia and to deficiency of arginine (excluding arginase deficiency). Arginine biosynthesis from citrulline is catalyzed by the urea cycle enzymes argininosuccinate synthetase (ASS) and arginase (ARG) [9]. The de novo biosynthesis of arginine from ammonia in the central nervous system (CNS) is absent, since the mitochondrial urea cycle enzymes carbamoylphosphate synthetase (CPS I) and ornithine transcarbamylase (OTC) are not synthesized in the brain. Conversely, citrulline is synthesized in CNS by nitric oxide synthases (NOS) [10].

Nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) possesses microbicidal, antiviral,

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antiparasitic and antitumour effects [11]. The functions of NO are mediated by its stimulation of soluble guanylate cyclase and a raise in cGMP. However, abnormal iNOS induction is implicated in the pathophysiology of wide range of human diseases [12]. NF- κ B proteins, a family of ubiquitous transcription factors, participate in immunological responsiveness, inflammatory processes and cell growth regulation [13].

Ammonia intoxication impairs vital central nervous system (CNS) functions such as energy metabolism [14], hemodynamics [15] and neurotransmission [16]. As an outcome of the balance between its ionic and gaseous forms, ammonia readily passes the blood–brain barrier (BBB) and accumulates in the brain [16]. Elevated arterial ammonia levels correlate closely with brain ammonia uptake; ammonia within the brain is then detoxified by astrocytes by glutamine synthetase (GS), resulting in the accrual of glutamine [17]. Glial fibrillary acid protein (GFAP, intracytoplasmic filamentous protein) alters astrocyte motility and shape by facilitating structural stability to astrocytic processes [18]. GFAP is a specific astrocytic marker under pathological conditions [18, 19]. The loss of GFAP is also associated with astrocytic swelling [19].

Flavonoids (polyphenolic compounds) are recognized to possess noticeable anti-inflammatory, antioxidant and anti-tumor activities [20]. Valuable protective influences of flavonoids have been documented in preventing brain ischaemia/reperfusion injury [21] in the inhibition of beta amyloid involved in Alzheimer's disease [22], and in cognitive declines [23]. Quercetin exerts immunomodulatory effects in the management of multiple sclerosis (MS), (an autoimmune disease), characterized by CNS inflammation and axonal injury [24]. Quercetin has been known to induce apoptosis, angiotensin converting enzyme II, inhibition of angiogenesis, modulation of the cell cycle and antimutagenic actions [25]. The objective of this study is to analyse the levels of expressions of molecular markers (urea cycle enzymes, neuronal and inflammatory) owing to treatment of quercetin in ammonium chloride (AC) induced hyperammonemic rats to assess its influence on anti inflammatory, cytoprotective and neuroprotective activities.

Materials and Methods

Chemicals

Quercetin and ammonium chloride were purchased from Sisco Research Laboratories, Mumbai, India. The primary polyclonal antibodies (anti-rat CPS-I, anti-rat OTC, anti-rat ASS, anti-rat ARG, anti-rat GS, anti-rat PAG, anti-rat IL-6, anti-rat NF- κ B, anti-rat iNOS and anti-rat β -actin) were

bought from Santa Cruz Biotech, CA, USA. The secondary antibodies were obtained from Bangalore Genei, Bangalore, India. All other chemicals were obtained from Sigma (St. Louis, Mo, USA).

Experimental Animals

Male Wistar rats (180–200 g) from Central Animal House, Annamalai University, Tamil Nadu, India were maintained at $24 \pm 2^\circ\text{C}$ on 12 h light/dark cycles. The rats were nourished with standard diet pellets (Hindustan Lever Ltd., Bangalore, India) and water *ad libitum*. Investigations were performed conferring to the ethical norms of the Institutional Animal Ethics Committee of Annamalai University (Approval No. 994, dated 02.05.2013). The rats were acclimatized for a week before starting the experiments.

Experimental Design

Hyperammonemia has been evoked in rats through intraperitoneal injection (i.p) of AC (100 mg/kg b.w) for 56 days [12]. Animals were randomly categorized into 4 groups each group containing six animals. Group I (control) rats, group II rats were administered with AC (100 mg/kg b.w), group III rats were treated with AC and quercetin (50 mg/kg b.w.) and group IV rats were treated with (50 mg/kg b.w.) quercetin alone orally.

Western Blot Analysis

The expression of CPS-I, OTC, ASS and ARG in liver and GS, PAG, IL-6, iNOS and NF- κ B in brain was investigated by western blotting. The liver and brain tissue sections were homogenized in chilled RIPA buffer ((Triton (1 %), SDS (0.1 %), 0.5 % deoxycholate (0.5 %), EDTA (1 mmol), Tris (20 mM, pH 7.6), NaCl (150 mmol), NaF (10 mmol), and 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF, 0.1 mmol)) and centrifuged at 13,000 rpm for 15 min at 5°C . The total protein was estimated [26]. Samples encompassing 50 μg of proteins were electrophoresed on a 10.5 % sodium dodecyl sulfate–polyacrylamide gel. The resolved proteins were blot transferred on to PVDF membrane (Millipore). The membranes were kept in blocking buffer with BSA (5 %) for 2.5 h to diminish non-specific binding. The membranes were then incubated with primary antibodies (in Tris-buffered saline and Tween-20 (0.04 %, TBST)), recognizing CPS-I, OTC, ASS, ARG and β -actin (1:1000 dilution), GS, and PAG (1:200) IL-6, NF- κ B and iNOS (1:500) in a quaking platform overnight at 5°C . Then, the membranes were incubated with the related secondary antibodies (anti-rat IgG conjugated to horseradish peroxidase) for 2 h. The

membranes were washed thrice with TBST for 20 min. The protein bands were visualized by an enhanced chemiluminescence method using an ECL kit (GenScript ECL kit, USA), scanned and analysed by Image J software (Bethesda, USA).

mRNA Expression of GFAP, sGC and ASS

Total RNA was separated and purified from the brain tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The cDNA was produced from 3 µg of total RNA treated with DNase by reverse transcription according to the kit instructions. Polymerase chain reaction was performed using PCR ready mix (Bangalore Genei). The PCR conditions for the housekeeping gene, glyceraldehydes 3-phosphate dehydrogenase (GAPDH) were 24 thermal cycles at 95 °C for 50, 56 °C for 50, and 73 °C for 70 s respectively and pursued by a final extension for 8 min at 73 °C. GFAP was subjected to 20 thermal cycles at 95 °C for 40, 60 °C for 40 s respectively, and 73 °C for 5 min followed by an a final extension at 73 °C for 4 min. sGC was subjected to 35 thermal cycles at 95 °C for 30, 55 °C for 50, and 73 °C for 60 s, respectively and pursued by an extension at 73 °C for 9 min (Table 1). Amplification reactions were accomplished in a 250 µM of dNTPs, 3.5 U of Taq DNA polymerase, in a volume of 50 µl containing 5 µl of cDNA and 150 pmol of primer (Bangalore Genei). PCR products (25 µl) were separated on a 1.8 % agarose gel containing ethidium bromide. Intensities of the bands were quantified using Gel Doc system (Bio-Rad, Hercules, CA, USA) and Quantity on 1-D analysis software (Bio-Rad). The result was represented as relative mRNA expression of target gene product/GAPDH.

Table 1 RT-PCR primer sequences for sGC, GFAP in brain and ASS in liver and positive control GAPDH in hyperammonemic and quercetin treated rats

Primer	Sequence
sGC	
Upstream	5'-ACCCTTATCTCCGCAGTCCT-3'
Downstream	5'-CGCCGTCAGGAGACTGATAC-3'
GFAP	
Upstream	5'-CGACCTTGAGTCCTTGCG-3'
Downstream	5'-CCGTCTTACCACGATGTT-3'
ASS	
Upstream	5'-ACGGAGCCAATAGAGGAGGT-3'
Downstream	5'-TGTCTTTCTTTGGGGGTGAC-3'
GAPDH	
Upstream	5'-ACCACAGTCCATGCCATCAC-3'
Downstream	5'-TCCACCACCCTGTTGCTGTA-3'

Statistical Analysis

All the data were expressed as mean $\pm$ SD ($n = 6$). Statistical significance was evaluated by one way analysis of variance (ANOVA) using SPSS 10.0 software. Duncan's Multiple Range Test (DMRT) was used to detect the significance between groups.

Results

Expression of Urea Cycle Enzymes in Liver and Astrocytic Markers Enzymes in Brain

The immunoblot expression of urea cycle enzymes—CPS-1, OTC, ASS and ARG of liver tissues and astrocytic markers (GS and PAG) in brain tissue showed down-regulated expression in AC-induced rats compared with controls. Treatment of quercetin to AC-treated rats significantly upregulated the expression of the urea cycle enzymes and astrocytic markers as compared with AC-induced rats (Figs. 1, 2).

Expression of Inflammatory Markers in Brain

The representative immunoblot of brain exhibited up-regulated expression of IL-6, iNOS and NF- κ B in AC-induced

Lane 1: Control, Lane 2: AC 100 mg/kg, Lane 3: Quercetin+AC, Lane 4: Quercetin

Fig. 1 Effect of quercetin on CPS-I, OTC, ASS and ARG protein expression in the liver tissue of control and AC-treated rats. Effect of quercetin on CPS-I, OTC, ASS and ARG protein band intensities scanned using a densitometer. β -Actin was used as internal standard to normalized the intensity of the protein expressions. The expressions from the six animals were quantified and their (mean $\pm$ SD) values were indicated in the graph * $p < 0.005$ compared with control rats; # $p < 0.05$ compared with AC-treated rats

Fig. 2 Effect of quercetin on GS and PAG protein expression in the brain tissue of control and AC-treated rats. Effect of quercetin on GS and PAG protein band intensities scanned using a densitometer. β -Actin was used as internal standard to normalize the intensity of the protein expressions. The expressions from the six animals were quantified and their (mean $\pm$ SD) values were indicated in the graph * $p < 0.005$ compared with control rats; # $p < 0.05$ compared with AC-treated rats

rats compared with control. Quercetin treatment to AC-induced rats significantly downregulated the expression of IL-6, iNOS and NF- κ B. There were no significant variations between control and quercetin (only) group (Fig. 3).

mRNA Expressions of sGC, GFAP in Brain and ASS in Liver

The mRNA expression of sGC and GFAP in brain and ASS in liver showed the levels were noticeable downregulation in hyperammonemic rats. Administration of quercetin concurrently reversed the expression of sGC, GFAP and ASS (Fig. 4).

Discussion

Liver failure causes impaired detoxification of ammonia and ammonia can get into the brain and alters its function. Detoxification of ammonia is chiefly accomplished by urea cycle in periportal hepatocytes. The inherited urea cycle defects (UCDs), i.e. the enzyme insufficiencies of CPS1, OTC, ASS, and ARG can all cause severe hyperammonemia. Our results documented the reduced expression of urea cycle enzymes such as CPS-1, OTC, ASS and ARG in AC administered rats in liver. It might be due to ammonia induced free radical generation and subsequent liver damage [12]. Quercetin treatment considerably improved the

Fig. 3 Effect of quercetin on IL-6, iNOS and NF- κ B protein expression in the brain tissue of control and AC-treated rats. Effect of quercetin on IL-6, iNOS and NF- κ B protein band intensities scanned using a densitometer. β -Actin was used as internal standard to normalize the intensity of the protein expressions. The expressions from the six animals were quantified and their (mean $\pm$ SD) values were indicated in the graph * $p < 0.005$ compared with control rats; # $p < 0.05$ compared with AC-treated rats

Fig. 4 sGC, GFAP in brain and ASS in liver mRNA expression in hyperammonemic and quercetin treated rats. Relative mRNA expression of sGC, GFAP and ASS. The results presented are the fold changes of target gene expression relative to the reference gene (GAPDH) expression. Bar graph shows the comparison among groups. The values are expressed as given as (mean $\pm$ SD) * $p < 0.005$ compared with control rats; # $p < 0.05$ compared with AC-treated rats

expression of urea cycle enzymes (CPS-1, OTC, ASS and ARG) in AC treated hyperammonemic rats possibly due to free-radical scavenging properties [27].

In brain, ammonia is detoxified via glutamine formation. The enzyme accountable for glutamine formation, GS, is elevated in astrocytes [28]. About 85 % of ammonia in blood is metabolized by GS in the astrocytes [29–31]. The results showed that the decreased expression of GS and PAG in brain AC induced rats. Treatment of quercetin significantly enhanced the expression of GS and PAG in hyperammonemic rats, which might be due to its free radical quenching effects and cytoprotection of cells of liver.

The function of glutamate-NO-cGMP pathway is altered in brain in vivo experimental models of chronic hyperammonemia. The acute ammonia intoxication affects various cerebral processes [32]. One of the main effects is excessive stimulation of NMDA receptors by glutamate which could lead to elevated intracellular Ca^{2+} levels, which bind to calmodulin and activate various enzymes, such as neuronal nitric oxide synthase (NOS), thus causing elevated nitric oxide (NO) formation which consecutively stimulates sGC resulting in the synthesis of cGMP, and finally leading to cerebral vasodilation [33].

Astrocytes release cytokines in response to injury or inflammation [1]. IL-6 is a pro-inflammatory and anti-inflammatory cytokine, can cross the blood brain barrier [34] and enhances fluid phase permeability of isolated brain endothelial cell in vitro, and increased the diffusion of ammonia into the astrocytes. The up-regulation of iNOS and elevated production of NO in astroglial cells is a main factor responsible for hyperammonemic conditions [35]. Astrocytic stimulation of iNOS may contribute to neuronal damage in hyperammonemia [36]. NF- κ B (primary regulator gene) is involved in the synthesis of pro-inflammatory cytokines and enzymes in the microglia nucleus. The blockade of NF- κ B transcriptional activity is also known to repress the iNOS and proinflammatory cytokines expressions [37–39]. Our results showed the increased expression of inflammatory markers, IL-6, iNOS and NF- κ B in (brain tissue) observed in AC-induced rats which could be mainly owing to hepatic damage. In contrast, quercetin treatment the expression of IL-6, iNOS and NF- κ B was significantly decreased in the hyperammonemic rats, suggesting the anti-oxidant, anti-inflammatory and neuroprotective effects of quercetin.

Further, our study documented that the mRNA expressions were significantly downregulated in brain astrocytic markers (GFAP and sGC) and liver urea cycle enzymes (ASS) observed in AC treated rats. These results corroborate the earlier reports on speeling effects of hyperammonemia or HE on the expression in astrocytic markers [40]. The ammonia concentration in brain (1–5 mM) in the primary cortical astrocytes had lead to a destabilization

mRNA expression of GFAP [41–43]. Our administration could also increase the ammonia level in brain to the range 1–5 mM [12]. Liver failure during hyperammonemia is well-known to diminish the level of sGC via NO in (brain) as documented in liver cirrhotic patients [33, 40]. The diminished levels of sGC by NO and hence the glutamate–NO–cGMP pathway could be involved in different neurological modifications found in HE. After the treatment of quercetin the mRNA expression of GFAP, sGC and ASS showed upregulated which might be due to quenching of NO levels, enrichment of antioxidants and amelioration of ammonia toxicity and finally leads to neuro cytoprotection.

The antihyperammonemic action of quercetin modulating, could be via increased and decreased the expressions of urea cycle enzymes (CPS-1, OTC, ASS and ARG), astrocytic, neuronal (GS and PAG) and inflammatory markers (IL-6, iNOS and NF- κ B).

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Compliance with Ethical Standards

Conflict of interest The authors declare that there is no conflict of interest.

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