

Modulation of interleukin-1 β mediated inflammatory response in human astrocytes by flavonoids: Implications in neuroprotection

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Abstract

The proinflammatory cytokine interleukin-1 β (IL-1 β) contributes to inflammation and neuronal death in CNS injuries and neurodegenerative pathologies, and astrocytes have been implicated as the primary mediators of IL-1 β induced neuronal death. As astrocytes play an important role in supporting the survival and functions of neurons, we investigated the effect of plant flavonoids quercetin and luteolin, with known anti-inflammatory properties in modulating the response of human astrocytes to IL-1 β for therapeutic intervention. Flavonoids significantly decreased the release of reactive oxygen species (ROS) from astrocytes stimulated with IL-1 β . This decrease was accompanied by an increase in expression of superoxide dismutase (SOD-1) and thioredoxin (TRX1)—mediators associated with protection against oxidative stress. Flavonoids not only modulated the expression of astrocytes specific molecules such as glial fibrillary acidic protein (GFAP), glutamine synthetase (GS), and ceruloplasmin (CP) both in the presence and absence of IL-1 β but also decreased the elevated levels of proinflammatory cytokine interleukin-6 (IL-6) and chemokines interleukin-8 (IL-8), interferon-inducible protein (IP-10), monocyte-chemoattractant protein-1 (MCP-1), and RANTES from IL-1 β activated astrocytes. Significant decrease in neuronal apoptosis was observed in neurons cultured in conditioned medium obtained from astrocytes treated with a combination of IL-1 β and flavonoids as compared to that treated with IL-1 β alone. Our result suggests that by (i) enhancing the potential of activated astrocytes to detoxify free radical, (ii) reducing the expression of proinflammatory cytokines and chemokines, and (iii) modulating expression of mediators associated with enhanced physiological activity of astrocyte in response to injury, flavonoids confer (iv) protection against IL-1 β induced astrocyte mediated neuronal damage.

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1. Introduction

Astrocytes play crucial role in the homeostasis and function of the central nervous system (CNS). Astrocytes provide the CNS with energy storage and generation, detoxification mechanisms and immune system modulation [3,14,31,43]. The CNS response to trauma, infection, inflammation, excitotoxicity, degenerative and dementia-type diseases manifests with accompanied reactive gliosis largely attributed to astrocytes [16,26]. Astrocytes play a prominent role in the metabolism of antioxidant glutathione (GSH) and defense against ROS [15]. *In vitro*

and *in vivo* studies have demonstrated that astrocyte–neuronal interactions are essential for the survival and normal function of neurons [7,12,27]. Indeed, astrocytes become activated in response to neurodegenerative diseases and injury, and depending upon the pathological stimulus their responses can be either adaptive or maladaptive [26].

The proinflammatory cytokine IL-1 β is a key mediator of inflammation and neuronal death in acute CNS injuries, such as stroke and brain trauma [1,5], and has been implicated in neurodegenerative diseases such as Alzheimer's disease [5,32]. Astrocytes are the primary mediator of IL-1 β induced neuronal cell death and this effect is dependent on the release of free radicals [42]. It has been hypothesized that factors released from IL-1 β activated astrocytes induces apoptosis and significant changes in metabolic activity of primary human neurons [13]. Moreover, IL-1 β not only up-regulates the death protein FasL

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in astrocytes, which may in turn mediate neuronal injury [17], but also induces astrocytes to release proinflammatory mediator prostaglandin that can adversely affect cell survival [11].

The antioxidant and anti-inflammatory activities [39] of natural polyphenolic compounds flavonoids have been extensively studied. An inhibition of IL-1 β induced IL-6 production was observed in human astrocytoma upon treatment with the flavonoid quercetin [8]. Quercetin has been reported to inhibit synthesis of heat shock protein (HSP70) and GFAP in injured astrocytes [44], and reactive oxygen species (ROS) generation in oxidatively stressed glial cells [19]. Luteolin, another flavonoid is known to inhibit inflammatory mediator expression [20]. As astrocytes play an important role in supporting the survival and functions of neurons [9], it is of great interest to find means of modulating astrocyte activation in CNS inflammatory responses for therapeutic intervention. Building upon the known anti-inflammatory properties of quercetin and luteolin, we investigated whether these flavonoids are capable of modulating IL-1 β mediated inflammatory response in human astrocytes and its consequences on human neurons.

2. Methods

2.1. Preparation of human astrocytes and neuronal cultures

Human fetal brains were obtained from Gurgaon Civil Hospital, India, with informed consent of mothers undergoing medical termination of first trimester pregnancies. The tissue from each specimen was processed separately and independently, as were subsequent cell cultures; there was no pooling of cells from distinct specimens. All the experimental protocols were reviewed and approved by the Institutional Human Ethics Committee. Human brain-derived progenitor cells from 8 to 16 weeks gestation fetal brain samples were grown as monolayers on poly-D-lysine in serum-free neurobasal media supplemented with 0.5% bovine serum albumin (BSA), 2 mM glutamine, N2 (Invitrogen, Carlsbad, CA), neural survival factor (Clonetics, Walkersville, MD), EGF (20 ng/ml) and bFGF (25 ng/ml), as described previously [25]. Astrocyte differentiation was initiated by changing to Eagle's MEM supplemented with 10% fetal calf serum and 2 mM glutamine; after 3 weeks 100% of the cells were immunoreactive for GFAP expression. Neuronal differentiation involved changing the growth factors in the progenitor media to brain-derived neurotrophic factor (BDNF, 10 ng/ml) and platelet-derived growth factor (PDGF)-A/B (10 ng/ml) for 3 weeks. More than 95% cells were positive for neuronal marker β III-tubulin (Covance, CA). All reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated.

2.2. Treatment of astrocyte cultures *in vitro*

Astrocytes differentiated from human brain-derived progenitor cells were maintained in MEM supplemented with 10% FBS. Prior to treatment, the cells were switched to serum-free media. After 12 h in serum-free media, media was replaced and cells were treated with fresh serum-free media containing either human IL-1 β (10 ng/ml, R&D Systems, MN) or flavonoids luteolin or quercetin (Sigma) at 25 μ M and 50 μ M concentrations or a combination of IL-1 β and flavonoids, for another 24 h. Vehicle (dimethyl sulphoxide, DMSO) treated astrocytes were used as controls. IL-1 β was used at 10 ng/ml concentration in all experiments. Following incubation, the supernatant was collected from the media for the different culture conditions and stored at -80°C for bead array whereas cells were processed for immunoblot analysis and ROS measurement as described later in this section.

2.3. Measurement of reactive oxygen species (ROS)

To monitor the level of ROS produced within cells, the cell permeable, nonpolar, H₂O₂-sensitive probe 5 (and 6)-chloromethyl-2',7'-dichlorodihydro-

fluorescein diacetate (DCFDA) was used [40]. The fluorescence intensity of intracellular DCFDA is a linear indicator of amount of H₂O₂ in the cells. To detect ROS (H₂O₂) accumulation, astrocytes were treated either with IL-1 β or flavonoids or combination of IL-1 β and flavonoids for 24 h. Control and treated astrocyte cultures were treated with DCFDA (5 μ M) for 30 min at 37 $^{\circ}\text{C}$, washed twice with PBS, lysed with lysis buffer and the protein obtained was used to measure relative fluorescence.

2.4. Western blotting analysis

Protein was isolated from astrocytes treated either with IL-1 β or flavonoids (quercetin or luteolin) or combination of IL-1 β and flavonoids or vehicle for 24 h, as described previously [18,41]. Briefly, following treatment the cells were washed twice with ice-cold PBS, then lysed in buffer containing 1% Triton-X-100, 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% Nonidet P (NP-40), 1 mM EDTA, 0.2% EGTA, 0.2% sodium orthovanadate and protease inhibitor cocktail [41]. DNA was sheared using a 24-gauge needle and the lysate was incubated on a rocking platform at 4 $^{\circ}\text{C}$ for 30 min before centrifugation at 10,000 $\times g$ for 15 min at 4 $^{\circ}\text{C}$ [4]. Protein concentrations were determined using Pierce BCA Protein Assay Kit (Pierce, Rockford, IL). Fifteen micrograms of each sample was electrophoresed on a 7–12% polyacrylamide gel and Western blot analysis was performed as described previously [18,24] with the following antibodies: mouse anti GS (Chemicon International), rabbit anti GFAP (Dako, Carpinteria, CA), rabbit anti-ceruloplasmin (Dako), rabbit anti GLT-1 (Alpha Diagnostic International, TX), rabbit anti SOD-1 (Santa Cruz Biotechnology, CA) and rabbit anti TRX1 (Lab Frontier, Seoul, Korea). Secondary antibodies were purchased from Vector Laboratories. After addition of chemiluminescence reagent (Amersham, Buckinghamshire, UK), the blots were exposed to Chemigenius, Bioimaging System (Syngene, Cambridge, UK) for developing and images were captured using Genesnap software (Syngene). The blots were stripped and reprobed with anti- β -tubulin (Santa Cruz Biotechnology) to determine equivalent loading as described [18]. Optical density measurements were made using Gentoools software (Syngene). Antibody against TRX1 and SOD-1 was a gift from Dr. Vijayalakshmi Ravindranath and Dr. Nihar Ranjan Jana, respectively, of National Brain Research Centre, Manesar, India.

2.5. Cytokine and apoptosis bead array

The Cytokine and Chemokine Bead Array Kit (BD CBA Human Inflammation Kit; BD Biosciences, NJ, USA) was used to quantitatively measure cytokine and chemokine expression levels in the medium collected from control and astrocytes treated either with IL-1 β or flavonoids (quercetin or luteolin) or a combination of IL-1 β and flavonoids as described [18]. The BD CBA Human Apoptosis Kit (BD Biosciences) was used to quantitatively measure the expression levels of Bcl-2 in the protein lysate collected from human neuronal cultures treated with conditioned media collected from control and astrocytes treated either with IL-1 β or flavonoids (quercetin or luteolin) or combination of IL-1 β and flavonoids. Using 50 μ l of human inflammation or apoptosis standard and sample dilutions, the assay was performed according to the manufacturer's instructions and analyzed on the FACS Calibur (Becton Dickinson). The beads coated with IL-6, IL-8, IP-10, MCP-1 and RANTES or Bcl-2 reacted with test lysates and standards, to which fluorescence dyes were then added. This method quantifies soluble particles, in this case molecules associated with inflammation and apoptosis using fluorescence-based detection mechanism. Analysis was performed using CBA software that allows the calculation of cytokine and Bcl-2 concentrations in unknown lysates [18].

2.6. Treatment of neurons and TUNEL assay

Human fetal neurons were plated at a density of 2×10^4 cells/well of 8-well chamber slides (Nunc, Denmark) in medium containing 1% FBS. After 24 h the medium was replaced with conditioned medium obtained from astrocytes treated with either IL-1 β or flavonoids or a combination of IL-1 β and flavonoids. Following incubation for another 24 h in the conditioned medium, dying neurons were identified using *in situ* Cell Death Detection Kit, TMR red (Roche, Germany) as described [18]. Briefly, neuronal cultures were fixed with 4% paraformaldehyde (PFA) in PBS and blocked with 4% BSA containing 0.02%

Triton-X-100. The fixed cells were then incubated in the TUNEL mix (terminal deoxynucleotidyl transferase in storage buffer and TMR red labeled-nucleotide mixture in reaction buffer) for 1 h at room temperature. The slides were mounted with Vectashield mounting media containing DAPI (Vector Laboratories).

2.7. Statistical analysis

All comparisons between groups were performed using two-tailed paired Student's *t*-test. All values of *P* less than 0.05 were taken as significant.

3. Results

3.1. Flavonoids reduces ROS production from IL-1 β treated human astrocytes

While treatment of astrocytes with luteolin or quercetin alone had no effect on ROS production, treatment with IL-1 β resulted in a significant two-fold ($P < 0.05$) increase in ROS production as compared to control (Fig. 1a and b). Interestingly, when luteolin was present in combination with IL-1 β , a marked decrease in ROS production was observed as compared to IL-1 β treated astrocytes (Fig. 1a). The ability of luteolin to decrease the enhanced production of ROS from IL-1 β treated astrocytes was concentration dependent. Though ROS production in astrocytes treated with IL-1 β and 25 μ M of luteolin was comparable to that observed in astrocytes treated with IL-1 β alone, ROS levels decreased significantly ($P < 0.05$) in astrocytes treated with IL-1 β and 50 μ M luteolin—with ROS production attaining levels comparable to control astrocytes (Fig. 1a). Quercetin was however more effective than luteolin in reducing the enhanced ROS levels in IL-1 β activated astrocytes, since ROS generation was significantly ($P < 0.05$) reduced to basal levels at both 25 μ M and 50 μ M concentrations of quercetin (Fig. 1b).

3.2. Flavonoids modulate the expression of molecules associated with oxidative stress

As both luteolin and quercetin were capable of decreasing the elevated levels of ROS in IL-1 β activated astrocytes and since SOD-1 and TRX1 plays an important role in the maintenance of cellular redox status, we performed Western blot analysis to investigate the status of these molecules in astrocytes treated

with luteolin or quercetin in the presence and absence of IL-1 β . While the levels of SOD-1 was comparable between control and astrocytes treated with 25 μ M luteolin, a significant ($P < 0.05$) two-fold increase in SOD-1 level was observed in astrocytes treated with either 50 μ M of luteolin (Fig. 2a) or IL-1 β (Fig. 2a and b). SOD-1 levels were also significantly ($P < 0.05$) elevated by 2- and 2.6-fold in astrocytes treated with IL-1 β and 25 μ M or 50 μ M of luteolin, respectively, as compared to control (Fig. 2a). Treatment with 50 μ M of quercetin alone resulted in a significant ($P < 0.05$) 2.3-fold increase in SOD-1 expression (Fig. 2b). A 3.4- and 3.8-fold increase in the level of SOD-1 was also observed in astrocytes treated with IL-1 β in the presence of 25 μ M or 50 μ M of quercetin, respectively (Fig. 2b), as compared to control.

Although treatment of astrocytes with 25 μ M of luteolin had no effect on TRX1 expression, a significant ($P < 0.05$) 4.8-fold increase in TRX1 level was observed in astrocytes treated with 50 μ M of luteolin, as compared to control (Fig. 2a). Interestingly, a significant ($P < 0.05$) two-fold increase in TRX1 level was observed in astrocytes treated with IL-1 β (Fig. 2a and b). Though this elevated TRX1 level in IL-1 β treated astrocytes was unaffected in the presence of 25 μ M of luteolin, a significant ($P < 0.05$) increase in TRX1 expression was observed in astrocytes treated with a combination of IL-1 β and 50 μ M of luteolin, as compared to astrocytes treated with IL-1 β alone (Fig. 2a). This increase was equivalent to a five-fold change in TRX1 levels as compared to the control. A significant ($P < 0.05$) 3.5-fold increase in TRX1 expression was observed in astrocytes treated with 50 μ M quercetin (Fig. 2b), while treatment with 25 μ M quercetin had no effect on TRX1 level. The two-fold increase in TRX1 expression observed in IL-1 β treated astrocytes was further elevated significantly ($P < 0.05$) by 3.5- and 4-fold in the presence of 25 μ M and 50 μ M quercetin, respectively, as compared to control (Fig. 2b).

3.3. Flavonoids modulate the expression of astrocyte specific markers both in the presence and absence of IL-1 β

To determine whether flavonoids are capable of modulating the neuroprotective functions of astrocytes, the levels of GFAP, GS, CP and GLT-1-marker, enzyme and transporter associated

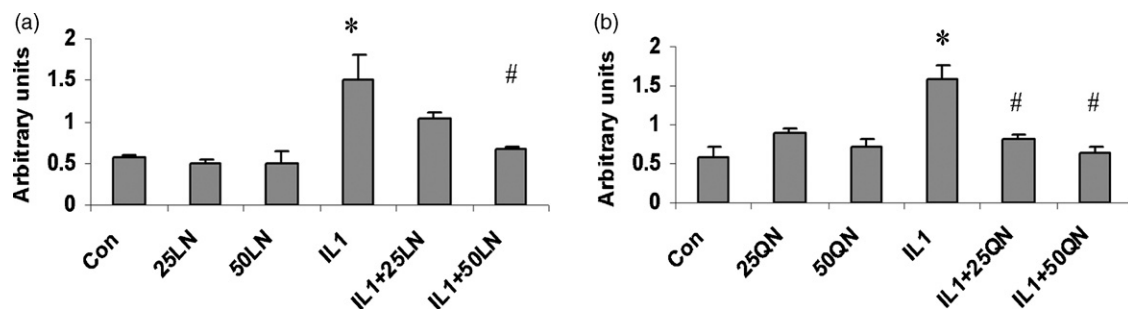


Fig. 1. Flavonoids confer protection against reactive oxygen species (ROS) generated in IL-1 β activated astrocytes. ROS production in human astrocytes treated with luteolin (a) or quercetin (b) in the presence and absence of IL-1 β (10 ng/ml) for 24 h. Human astrocytes were treated with 25 μ M and 50 μ M of luteolin or quercetin either alone or in combination with IL-1 β for 24 h. Following treatment protein was isolated and from equal amount of protein ROS levels were measured using H₂O₂-sensitive probe DCFDA. The graph indicates the level of ROS generated under different culture conditions. Error bars represent the means \pm S.E.M. from three individual experiments. *Significant change from control $P < 0.05$; #significant change from IL-1 β treated astrocytes $P < 0.05$. LN and QN represent luteolin and quercetin, respectively.

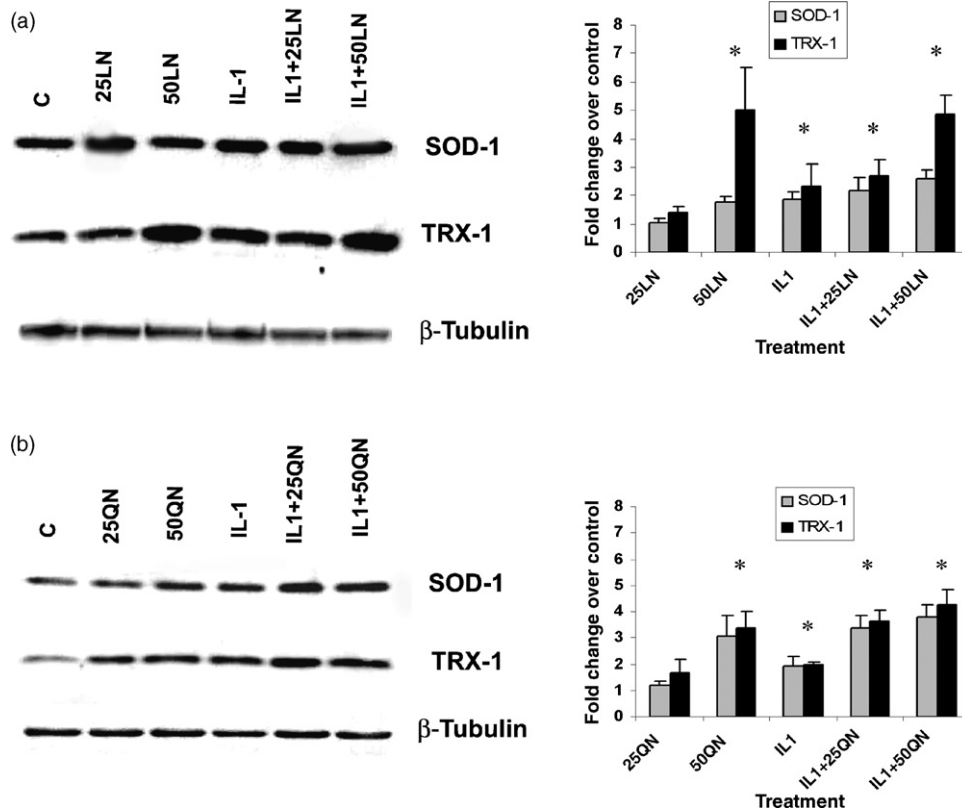


Fig. 2. Flavonoids effect on the expression of molecules associated with oxidative stress. SOD-1 and TRX1 expressions were analyzed by Western blot with protein samples extracted from astrocytes treated with 25 μ M and 50 μ M of luteolin (a) or quercetin (b) either alone or in combination with IL-1 β (10 ng/ml) for 24 h. Blots were reprobbed for β tubulin to establish equal protein loading. Vehicle treated astrocytes were used as controls. The fold change in expression of SOD-1 and TRX1 in astrocytes treated with flavonoids in the presence and absence of IL-1 β over the control is shown. Densitometric measurements were performed on individual immunoblots for each antibody tested and values represent the means \pm S.E.M. from three individual experiments. *Significant change from control $P < 0.05$. LN and QN represent luteolin and quercetin, respectively.

with physiological functions of astrocytes were analyzed. While the levels of GFAP between control and astrocytes treated with either luteolin or quercetin were comparable, a significant ($P < 0.05$) two-fold increase in GFAP expression was observed in IL-1 β treated astrocytes (Fig. 3a and b). Although, this elevated level of GFAP was unaffected in astrocytes treated with IL-1 β and 25 μ M of luteolin or quercetin, a significant ($P < 0.05$) decrease in GFAP expression was observed in astrocytes treated with IL-1 β and 50 μ M of luteolin or quercetin, as compared to astrocytes treated with IL-1 β alone (Fig. 3a and b).

As the capacity of astrocytes to reduce extracellular levels of glutamate can dramatically impact the extent of neuronal damage after an insult [29] and since glutamate is predominantly converted to glutamine in astrocytes by the enzyme glutamine synthetase (GS) produced almost exclusively by astrocytes [10], Western blot analysis was performed to detect the expression of GS in astrocytes treated with flavonoids alone, or IL-1 β or IL-1 β in the presence of flavonoids. Though treatment with 25 μ M of either luteolin or quercetin had no effect of astrocytic GS expression, a significant ($P < 0.05$) 2.5- and 3.2-fold increase in GS level was observed in astrocytes treated with 50 μ M of luteolin or quercetin, respectively, as compared to control (Fig. 3a and b). While there was a trend towards decrease in GS levels

in IL-1 β treated astrocytes, a significant ($P < 0.05$) increase in GS levels was observed in astrocytes treated with a combination of IL-1 β and 25 μ M and 50 μ M of luteolin or quercetin, as compared to control (Fig. 3a and b).

As the expression of ceruloplasmin (CP)—a multifunctional protein that serves as an amine oxidase, an antioxidant, a ferroxidase [36], is increased in astrocytes as a consequence of IL-1 β stimulation [24], we investigated whether flavonoids could effect ceruloplasmin levels in the presence and absence of IL-1 β . While luteolin and quercetin had no effect on CP expression, a significant ($P < 0.05$) \sim 1.6-fold increase in CP expression was observed in IL-1 β treated astrocytes, as compared to control (Fig. 3a and b). The elevated CP level in IL-1 β treated astrocytes was unaffected by the presence of luteolin or quercetin, since CP levels were comparable between astrocytes treated with a combination of IL-1 β and luteolin or quercetin and astrocytes treated with IL-1 β alone (Fig. 3a and b). As glutamate transporters are essential to transport extracellular glutamate into astrocytes, we determined the expression levels of glutamate transporter GLT-1 in astrocytes treated with either flavonoids alone, or IL-1 β or IL-1 β in the presence of flavonoids for 24 h. The expression of GLT-1 was unaffected by IL-1 β treatment either in the absence and presence of either flavonoids, as compared to control (data not shown).

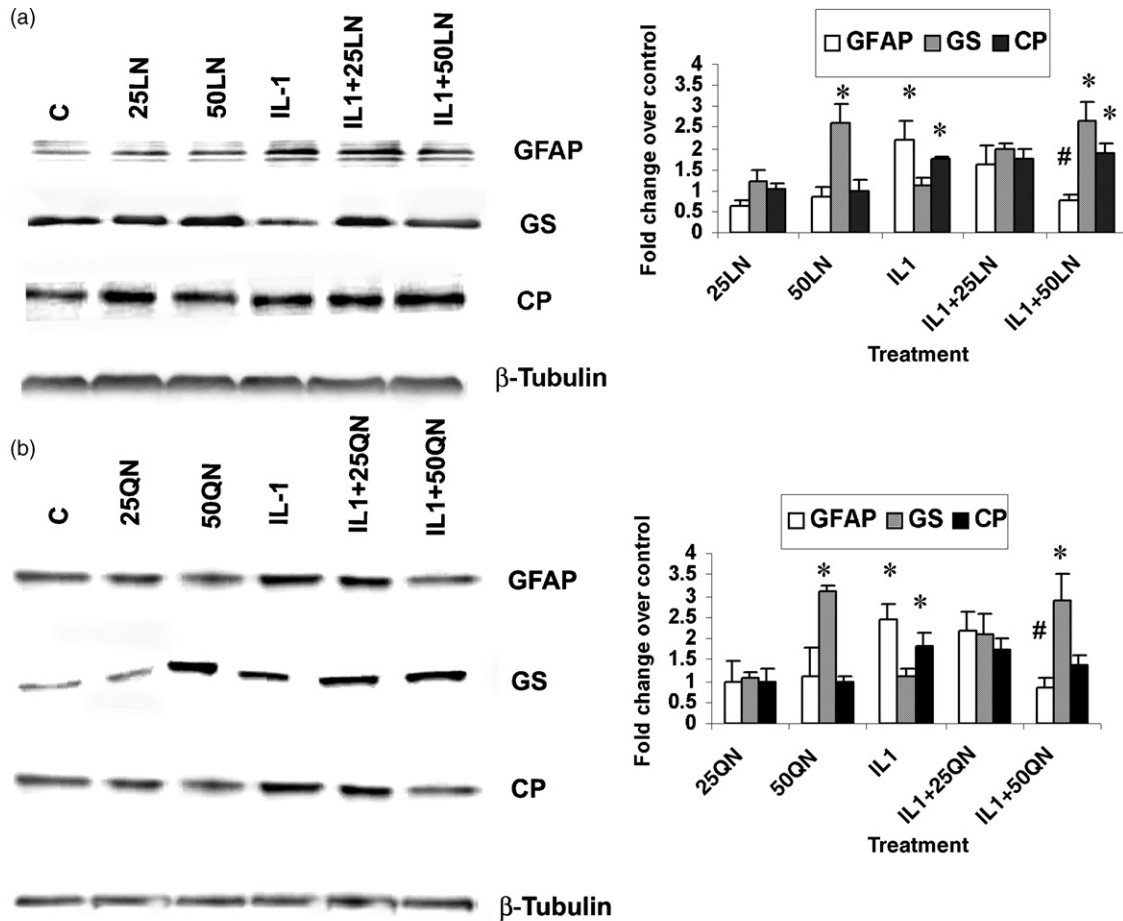


Fig. 3. Flavonoids modulate the expression of astrocyte specific markers in the presence or absence of IL-1 β . Western blot showing the expression of GFAP, GS, and CP in protein samples from astrocytes treated with IL-1 β (10 ng/ml) in the presence or absence 25 μ M and 50 μ M of luteolin (a) and quercetin (b) for 24 h. Data are representative of results obtained from three independent experiments. Densitometric measurements were performed on individual immunoblots for each antibody tested and values represent the means \pm S.E.M. from three individual experiments. *Significant change from control $P < 0.05$; # significant change from IL-1 β treated astrocytes $P < 0.05$. LN and QN represent luteolin and quercetin, respectively.

3.4. Flavonoids inhibits the release of proinflammatory cytokines and chemokines from IL-1 β activated human astrocytes

Since activated astrocytes secrete various proinflammatory cytokines and chemokines [22], cytokine bead array (CBA) was performed to investigate the profile of proinflammatory mediators in astrocytes treated with IL-1 β in the presence and absence of 50 μ M of luteolin or quercetin. There was a significant 10-fold increase in IL-6 expression ($P < 0.05$) in IL-1 β treated astrocytes, as compared to the control (Fig. 4a). Importantly, the enhanced IL-6 level observed in IL-1 β treated astrocytes was significantly reduced ($P < 0.05$) to control levels, when astrocytes were treated with a combination of IL-1 β and luteolin or quercetin (Fig. 4a). The significant 20-, 6-, 5- and 4-fold induction in chemokines IL-8, IP-10, MCP-1, and RANTES, respectively, observed in IL-1 β treated astrocytes as compared to control, was dramatically reduced to basal level when luteolin or quercetin was present in IL-1 β treated cultures (Fig. 4b–e).

3.5. Flavonoids prevents IL-1 β induced and astrocyte mediated neuronal death

We next performed TUNEL staining to investigate whether flavonoids are capable of protecting human neuronal cultures against astrocyte mediated IL-1 β induced neurotoxicity. The conditioned media collected from astrocytes treated with different combinations of IL-1 β and 50 μ M flavonoids was used to treat human neuronal cultures. There was no difference in the percentage of apoptotic cell in neuronal cultures treated with conditioned medium obtained from astrocytes that had been treated with either luteolin or quercetin or vehicle alone (Fig. 5a and b). However, a significant ($P < 0.05$) three-fold increase in TUNEL positive apoptotic cell was observed when neurons were cultured in conditioned medium obtained from astrocytes treated with IL-1 β (Fig. 5a and b). Interestingly, the number of apoptotic cells observed when neuronal cells were treated with conditioned media derived from astrocytes treated with a combination of IL-1 β and quercetin or luteolin, was comparable to the control. Treatment of neurons with quercetin or luteolin alone had

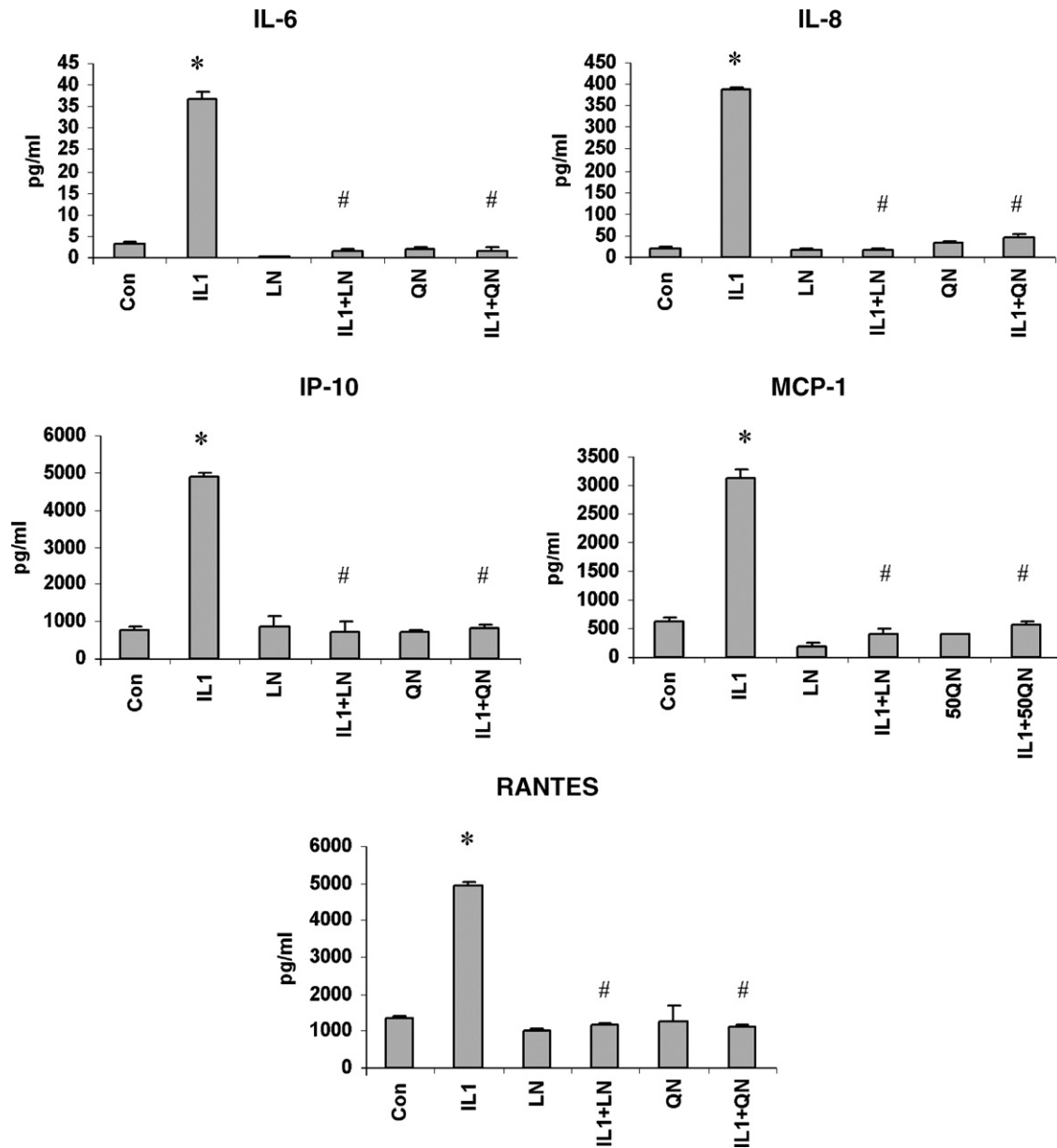


Fig. 4. Flavonoids affect the release of proinflammatory cytokine and chemokines from IL-1 β stimulated human astrocytes. Expression of IL-6, IL-8, IP-10, MCP-1 and RANTES from human astrocytes treated with 50 μ M of luteolin or quercetin either alone or in combination with IL-1 β for 24 h, as observed by cytokine bead array. The level of IL-6 (a), IL-8 (b), IP-10 (c), MCP-1 (d) and RANTES (e), elevated in IL-1 β treated astrocytes as compared to control, was suppressed when astrocytes were treated with a combination of IL-1 β and 50 μ M of either luteolin or quercetin. Values represent means \pm S.E.M. from three individual experiments. *Significant change from control $P < 0.05$; #significant change from IL-1 β treated astrocytes $P < 0.05$.

no effect on viability as the number of viable neurons was comparable between control and flavonoid treated samples (data not shown).

We next performed apoptosis bead array to determine the status of pro-survival molecule Bcl-2 in neuronal cultures treated with conditioned medium collected from astrocytes treated with 50 μ M flavonoids in the presence or absence of IL-1 β . Bcl-2 expression was comparable between neuronal cultures treated with conditioned media from astrocytes cultured in the presence of either luteolin or quercetin or vehicle (Fig. 5c). However, the expression of Bcl-2 was significantly reduced (0.6-fold decrease) in neuronal cultures treated with conditioned media

from astrocytes treated with IL-1 β (Fig. 5c). On the other hand, when neurons were grown in conditioned medium from astrocyte treated with IL-1 β and luteolin or quercetin, the Bcl-2 levels were significantly elevated to the levels observed in neurons grown in conditioned medium from control (Fig. 5c).

4. Discussion

Astrocytes play a significant role in neurodegenerative diseases and IL-1 β coordinates many of the initial and subsequent responses of astrocytes to injury [22,26]. IL-1 β induces the expression of many inflammatory mediators that can

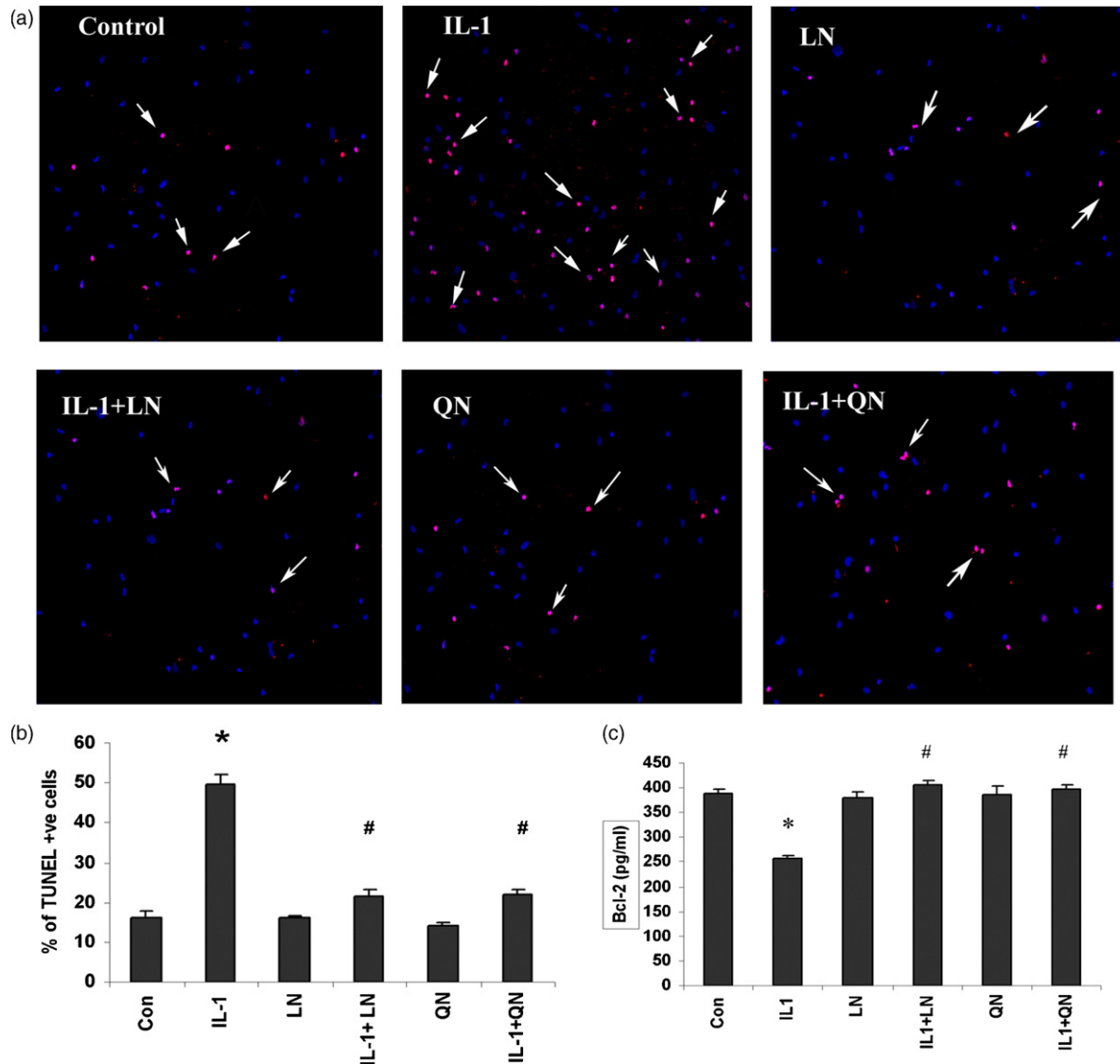


Fig. 5. Inhibition of astrocyte mediated IL-1 β induced neuronal apoptosis by flavonoids. (a) TUNEL or cell death assay of human neuronal cultures showing TUNEL positive cells (red) co-localized with DAPI, when neurons were cultured in conditioned media obtained from astrocytes treated with either luteolin or quercetin in the presence or absence of IL-1 β . (b) The graph represents the percentage of TUNEL positive neuronal cells under the different culture conditions described in (a), as counted from multiple fields. Values represent the means \pm S.E.M. from two independent experiments (*significant change from control astrocytes $P < 0.005$; #significant change from supernatant obtained from IL-1 β treated astrocytes, $P < 0.005$). (c) Bcl-2 levels in human neurons treated with conditioned media from astrocytes treated with flavonoids in the presence and absence of IL-1 β . Bcl-2 from human astrocytes treated with 50 μ M of luteolin or quercetin alone or in combination with IL-1 β , as observed by apoptosis bead array. Values represent means \pm S.E.M. from two individual experiments performed in triplicate. *Significant change from control $P < 0.05$; #significant change from IL-1 β treated astrocytes $P < 0.05$. LN and QN represent luteolin and quercetin, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

cause undesirable effects [5]. It is known that IL-1 β induces neuronal death through actions on astrocytes and IL-1 β has no direct effect on the viability of neurons [42]. Since the anti-inflammatory role of flavonoids is well documented [23], this study was designed to investigate the prophylactic effect of the flavonoids quercetin and luteolin in modulating IL-1 β induced inflammatory response in human astrocytes. Our studies indicated that treatment with the flavonoids luteolin and quercetin (i) reduces oxidative stress in IL-1 β activated astrocytes by decreasing ROS production while (ii) concomitantly increasing levels of antioxidants SOD-1 and TRX1. The flavonoids not only modulated (iii) the expression of astrocyte specific molecules

GFAP, GS and CP but also (iv) suppressed the production of proinflammatory cytokines and chemokines IL-6, IL-8, IP-10, MCP-1 and RANTES from IL-1 β stimulated astrocytes. Importantly, mediators released from IL-1 β activated astrocytes adversely affected neuronal viability by down regulating Bcl-2 expression and flavonoids were capable of rescuing neurons from IL-1 β induced astrocyte mediated neurotoxicity.

Generation of ROS with the accumulation of oxidative damage has been implicated in neurodegenerative diseases. Astrocytes play a prominent role in the metabolism of antioxidant glutathione (GSH) and defense against ROS [15], and inflammation decreases the expression of antioxidant SOD in

astrocytes [35]. Over expression of TRX1 with known antioxidant and anti-inflammatory effects, has been shown to suppress both oxidative stress and IL-6 production [34]. Increased SOD-1 and TRX1 levels in IL-1 β activated astrocytes reflect an attempt made by the cells to scavenge excess ROS generated following inflammation. By further increasing the elevated SOD-1 and TRX1 levels in IL-1 β treated astrocytes, flavonoids ensure that these molecules are readily available to meet the increased demand in an inflamed environment.

Reactive astrogliosis, characterized by increased GFAP content, is a hallmark associated with vigorous response of astrocytes to diverse insults including inflammation [38]. Moreover, rapid astrogliosis leads to the formation of glial scar that inhibit axonal growth [37]. It is tempting to speculate that by decreasing GFAP production and increasing GS expression in IL-1 β activated astrocytes, flavonoids reduces reactive gliosis and possibly modulates the capacity of astrocytes to handle elevated glutamate under inflammatory conditions. Protective response elicited by IL-1 β through increased CP expression in astrocytes, was maintained in the presence of quercetin and luteolin.

Human astrocytes produces a wide variety of chemokines and cytokines upon exposure to proinflammatory stimuli [30]. IL-1 β primed astrocytes release proinflammatory cytokine IL-6 which can again establish a feed forward cycle of inflammation [6]. IL-1 β induces astrocytes to produce IL-8 that is responsible for leucocytosis associated with inflammation [2]. Chemokines IP-10, IL-8, MCP-1 and RANTES released from IL-1 β activated astrocytes [33] enhances the severity of neuronal injury [22] and serves as prime mediator of astrogliosis [28]. Although the net effect of these proinflammatory mediators is to stimulate the production of molecules that amplifies the response of astrocytes to inflammation, a dysregulated innate immune response would be deleterious. The ability of flavonoids to inhibit the release of IL-8, IP-10, RANTES and MCP-1 from IL-1 β activated astrocyte might reflect an effect to negate the deleterious effect of inflammation.

Considering our findings and previous report that astrocytes are the primary mediators of IL-1 β induced neuronal cell death [42], it is evident that luteolin and quercetin induces neuroprotection primarily through the inhibition of inflammatory mediators, ROS and modulation of astrocyte markers that are associated with response to injury. Not only are flavonoids able to traverse the blood brain barrier (BBB) *in vivo* [45], but its low toxicity makes the margin of safety for its therapeutic use large [21]. That flavonoids mediate important compensatory responses in inflamed astrocytes to bring about the resolution of inflammation and to restore homeostasis, raises the possibility that flavonoids might serve as an effective therapeutic strategy where inflammatory responses of astrocytes are believed to contribute to neuronal death.

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