Chapter- V

To evaluate anti-inflammatory role of quercetin and DHA on LPS activated astrocytes.

5.1 Introduction

Immune responses in CNS were mainly attributed to microglia (Rivest 2009) but the studies in the last decade have shown highly noteworthy contribution of astrocytes in causing inflammation (Carpentier et al. 2005, Dong and Benveniste 2001, Marinelli et al. 2015, Colombo and Farina 2016). Astrocytic release of pro inflammatory mediators that not only exert paracrine effects in neighbouring cells, but also promote autocrine effects thus grounds higher astroglial reactivity in response to inflammation. Pro-inflammatory markers are detrimental when expressed in higher amount (Van Wagoner and Benveniste 1999, Colombo and Farina 2016) and various CNS diseases have already been documented with higher inflammation such as Multiple sclerosis, Parkinson's disease, Alzheimer's disease, and AIDS dementia (Allan and Rothwell 2003b, Li et al. 2011, Maragakis and Rothstein 2006a, Sharma et al. 2010, Chitnis and Weiner 2017, Allan and Rothwell 2003a). Overall, inflammation is one of the prime factors for causing CNS diseases (Chitnis and Weiner 2017, Jeyakumar et al. 2003, Lucas, Rothwell, and Gibson 2006) and astrocytes have the prodigious potential to impact detrimentally on surrounding neural and non-neural cells through inflammation (Colombo and Farina 2016).

Astrocytes being the bridge between the immune system and nervous system (Dong and Benveniste, 2001; Carpentier et al., 2005; Marinelli et al., 2015), can express Toll like receptors TLRs (1-9) in humans and in rodents at different levels (Bowman et al.,2003; Kielian, 2006; Jack et al., 2009). Previous reports on human and rodent astrocytes have documented that Toll like receptor TLR-4 signalling is severely affected in some of the acute, chronic and neurodevelopmental disorders (Kielian, 2006; Buchanan et al., 2010; Henneberger and Steinhäuser, 2016). Bacterial lipopolysachharide (LPS), an endotoxin, the main ligand of TLR-4 receptor, causes activation of downstream MyD88 dependent and MyD88 independent pathways in turn increases release of cytokines, chemokines and growth factors (Jack et al., 2005; Krasowska-Zoladek et al., 2007; Gorina et al.,2011).

Pro-inflammatory molecules IL-1β, IL-6, TNF-α, and COX-2 are up regulated in neurodegenerative diseases and down regulating these have shown ameliorating the diseases in *in vivo* models (Tansey and Wyss-Coray 2008, Lucas, Rothwell, and Gibson 2006, Chitnis and Weiner 2017, Selmaj 1992, Allan and Rothwell 2003a). Hemeoxygenase-1 (HO-1) has also been found to be down-regulated in CNS disorders (S Ambegaokar and L Kolson 2014). HO-1 has been also shown to down regulate pro-inflammatory markers and help alleviate inflammation mediated damage (Chen et al. 2013, Liu et al. 2016, Jazwa and Cuadrado 2010).

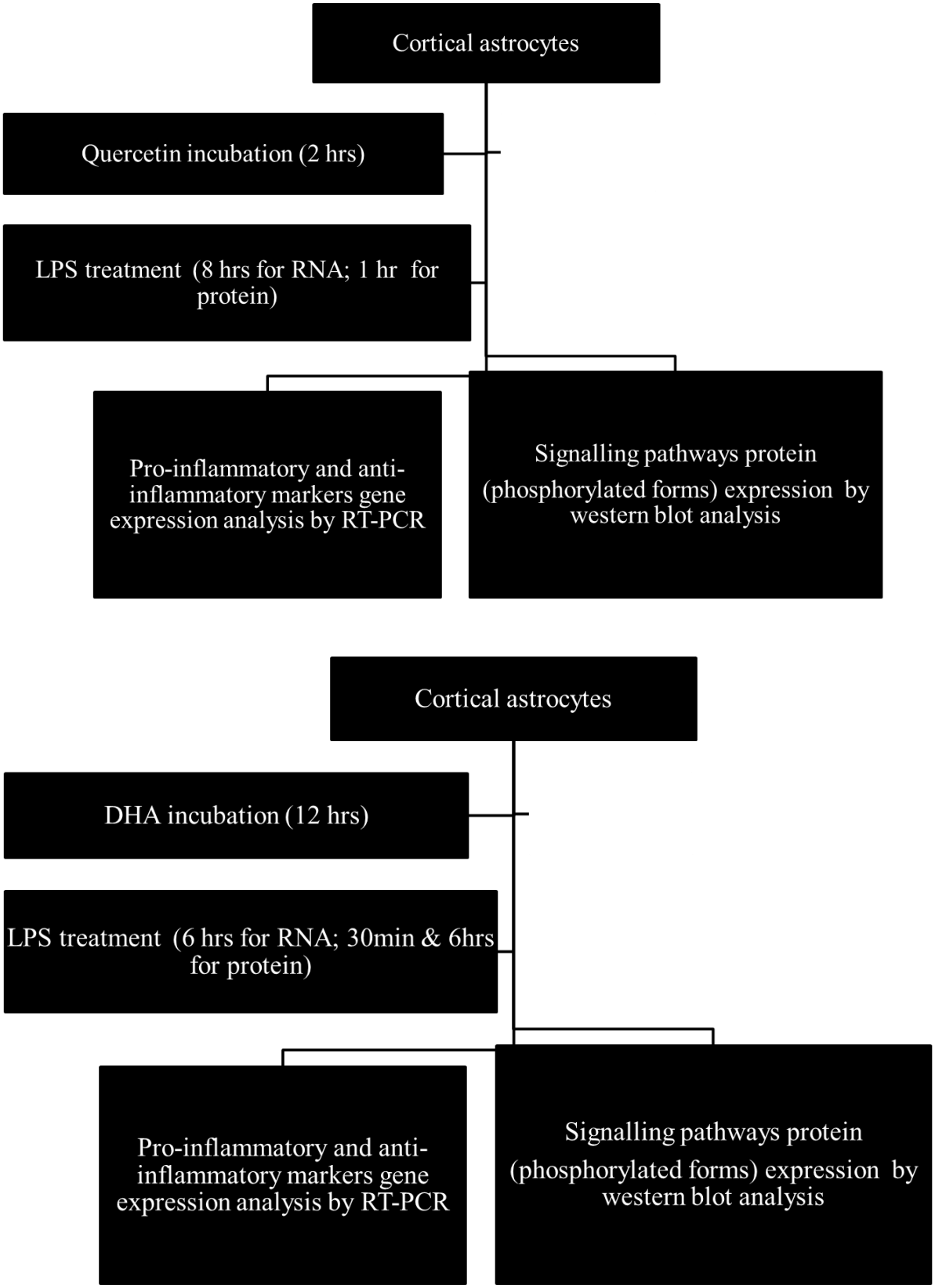
Previously, the reports have shown anti-inflammatory effects of quercetin by stimulating HO-1 protein expression, increasing phosphorylation of p38 MAPK and reducing iNOS, IL-6, COX-2 expressions in LPS induced microglial cells (Sun et al. 2015, Bureau, Longpré, and Martinoli 2008, Chen et al. 2005, Kao et al. 2010, Yang et al. 2017); lowering IL-1β,IL-6, TNF-α and iNOS genes expressions by reducing the activation of ERK1/2 kinase and p38 kinase in macrophage RAW264.7 cell line (Cho et al. 2003); reducing iNOS expression mediated by suppression of p38 and JNK phosphorylation in cardiomyoblasts (Angeloni and Hrelia 2012); suppressing cytokine release with increased HO-1 in muscle cells and decreased reactivity of glial cells (Borghi et al. 2016); lowering IL-1β,IL-6, TNF-α and increased HO-1 expressions in lung cells(Takashima et al. 2014); and modulating NF-κB pathway in intestinal epithelial cells (Ruiz et al. 2007). Moreover, quercetin (50μM) has been found to be down-regulating pro-inflammatory cytokine (IL-6) and chemokines (IL-8, IP-10, MCP-1 and RANTES) release and reducing GFAP content in IL-1β induced human astrocytes (Sharma et al. 2007) but the effects of quercetin on other important pro-inflammatory markers and underlying mechanism in astrocytes has not been evaluated. Quercetin has been already known to down regulate expression of certain pro-inflammatory cytokines in microglial cells at 0.1-20μM (Bournival et al. 2012, Mrvová et al. 2015, Sun et al. 2015, Yang et al. 2017) but no effect of lower concentrations on astrocytes has been observed. In fact, quercetin concentrations (25 & 50 μM) application exhibited only subtle fold difference in cytokines release in inflammation induced astrocytes (Sharma et al. 2007). Thus, there are limited observations showing effect of quercetin on inflammation status in astrocytes.

Docosahexaenoic acid (DHA) has also been documented to have anti-inflammatory effects studied in various cell types *in vitro* and *in vivo* reviewed by (Calder 2008, Dyall 2015, Teng et al. 2014, Chapkin et al. 2009, Calder 2009, Ibarguren, López, and Escribá 2014). Other than its modulatory effects through its impact on the cell membrane lipid composition, it has been found

to regulate intracellular signalling pathways (Le Guen et al. 2015, Teng et al. 2014, Ibarguren, López, and Escribá 2014, Russell and Bürgin-Maunder 2012). TLR-4 signalling pathway has been found to be blocked by DHA which could be owing to either the down regulation of NADPH oxidase production as this enzyme induces ROS generation (necessary for TLR-4 signalling) or the incorporation of DHA into lipid membrane which disrupts the translocation of TLR-4 into lipid raft, thus inhibiting the TLR-4 signalling pathway (Teng et al. 2014). Some of the intracellular signalling pathways (AMPK, NF-κB, PPAR, MAPK) play a pivotal role in inflammation that have also been reported to be altered by DHA administration (Le Guen et al. 2015, Teng et al. 2014, Russell and Bürgin-Maunder 2012, Gorjão et al. 2009). In palmitic acid induced astrocytes, Il-6 and TNF-α (cytokines) release was lowered in DHA (10-100μM) treated cells (Gupta et al. 2012). Studies focusing on anti-inflammatory effects are mostly done using Omega-3 PUFAs (EPA and DHA both) or its metabolites (Resolvin & neuroprotectin) and so role of DHA alone as an anti-inflammatory agent needs further attention. On the whole, DHA has also been known to play anti-inflammatory role but evidences on its effect on astrocytic inflammatory molecules expression and underlying mechanisms are insufficient.

Thus, this study was aimed to evaluate the anti-inflammatory role of different micromolar concentrations of quercetin (QH) and Docosahexaenoic acid (DHA) in astrocytes. To study the same, inflammation model was established by treating astrocytes with lipopolysachharide (LPS- cell wall component of E.coli). Effect of quercetin (25,50,100,200μM) and DHA (25, 100μM) were ascertained on inflammatory markers- IL-1β, IL-6, TNF-α, COX-2, HO-1, and TLR-4 genes expressions by semi quantitative RT-PCR and signalling molecules pERK1/2, phospho p38, and p-IκB-α by western blot.

5.2 Experimental design



5.3 Results & discussion

5.3.1 Pattern of inflammatory gene expression in response to LPS in cortical astrocytes

In this study, the rat brain cortical astrocytes were exposed to LPS (1μg/ml) for 30 min, 4 hrs, 8 hrs, 12 hrs and 24 hrs. TLR-4 is the major receptor responsible for mediating effects of lipopolysachharide (LPS)- a cell wall component of gram negative bacteria and is expressed in astrocytes (Bowman et al. 2003, Gorina et al. 2011, Nakano et al. 2015). TLR-4 mRNA level did not change in LPS treated groups at any time point in comparison to control (Fig.5.1.f) as documented in the other reports (Marinelli et al. 2015, Bellini et al. 2011, Santos-Galindo et al. 2011, Tarassishin, Suh, and Lee 2014). Elevated pro inflammatory cytokines (IL-1β, IL-6, TNF-α) and COX-2 enzyme are involved in induction of inflammation and triggering adaptive immune responses whereas increased hemeoxygenase-1 can suppress inflammatory responses in astrocytes during the pathogenesis of CNS diseases (Farina, Aloisi, and Meinl 2007, Merrill and Chen 1991, Lin et al. 2011). LPS exposure (1μg/ml) has been shown to exhibit substantial increase in inflammatory markers in macrophage (Cho et al. 2003) whereas reached plateau state in astrocytes (Krasowska-Zoladek et al. 2007) at 24 hrs LPS exposure. But in the current study, diminishing to basal levels of IL-1β, IL-6, TNF-α and COX-2 mRNAs at 24 hrs were observed (Fig.5.1.a-d). Also, the LPS induced HO-1 expression was observed at early time points which decreased at 8 hrs and 12 hrs. This could be due to the inherent feedback mechanisms that regulate pro-inflammatory responses in astrocytes. IL-1β, IL-6, TNF-α, and COX-2 mRNA levels increased at 30 min and persisted till 12 hrs (Fig.5.1). The highest cytokines and COX-2 expressions and lowest HO-1 expression were observed at 8 hrs of LPS exposure, henceforth this time point was considered to examine effects of QH doses (Fig.5.1).

5.3.2 Quercetin inhibits pro-inflammatory markers and promotes anti-inflammatory marker gene expression in LPS activated astrocytes

To assess the effect of quercetin on gene expression, QH was incubated for 2 hrs followed by 8 hrs of LPS exposure. QH was incubated before the LPS insult as QH higher concentration in medium might interfere with LPS binding to TLR-4 receptor. TLR related pathways are known to be operative in many of the neurological diseases (Buchanan et al. 2010, Henneberger and Steinhäuser 2016, Kielian 2006). TLR-4 silencing by siRNA has been shown to suppress the LPS responses in astrocytes (Tarassishin, Suh, and Lee 2014).Hence, the TLR-4 gene expression was examined in QH treated groups to determine if QH exerts any effects through modulating TLR-4

gene expression and no change was observed in QH treated groups (Fig. 5.2.f). Pro-inflammatory cytokines (IL-1β, IL-6) mRNA levels reduced significantly in 50 μM QH treated cells whereas, cytokines (IL-6, TNF-α) mRNA levels reduced significantly in 100 μM QH treated cells but they remained unaltered in 25 μM or 200 μM QH treated LPS activated astrocytes compared with LPS alone (Fig.5.2). These quercetin dose dependent gene expression patterns were quite different from the observation in macrophage cell line (Cho et al. 2003). COX-2 mRNA level was decreased in QH (50, 100 and 200 μM) concentrations and HO-1 level was increased in QH (25, 100, 200 μM) concentrations treated LPS activated astrocytes. Thus, pre-treatment of 100 μM QH dose was effective in favourable regulation of almost all the evaluated markers which is in line with the previous studies in macrophage cell line (Cho et al. 2003).

5.3.3 Quercetin modulates activation of MAPK and NF-κB pathways in dose dependent manner

Involvement of multiple signalling molecules operated during inflammation makes it the complex mechanism. Inflammation in astrocytes is reported to be via MAPK, NF-κB, JAK/STAT and AP-1 pathways (Gorina et al. 2011). Bacterial lipopolysaccharide (LPS) binding to TLR-4 activates MyD88 adaptor which in turn causes early activation of NF-κB and MyD88 independent activation of MAPK. p38 regulates phosphorylation of Stat1 and the transcription activity of NF-κB thereby influences LPS induced gene expression in astrocytes (Gorina et al. 2011). The pro-inflammatory markers under p38 regulation are IL-1β, IL-6, TNF-α, MCP-1, COX-2, iNOS (Lu et al. 2010, Font-Nieves et al. 2012). In the current study, phospho p38 level was found to be lowered in QH (50, 100 and 200 μM) treated cells compared to LPS alone (Fig. 5.3). The mRNA levels of IL-1β, IL-6, TNF-α, COX-2 were down-regulated in QH (50, 100 or 200 μM) treated cells but none of them were affected by QH (25μM) which is concomitant with the phospho p38 expression. Quercetin caused increased phosphorylation of p38 MAPK which also controls Nrf2 pathway at lower micromolar doses in LPS induced microglial BV-2 cell line (Sun et al. 2015). In this study, higher phosphorylated p38 expression and also up regulated hemeoxygenase-1 (Nrf2 pathway product) in QH (25μM) dose treated cells were observed. However, the significantly increased HO-1 mRNAs in higher QH doses treated cells suggest more activation of Nrf2 pathway or involvement of other pathways also in regulation of HO-1 in astrocytes via quercetin.

LPS has been documented to cause delayed ERK1/2 activation after 2 hrs (Gorina et al. 2011). In this study, no activation of ERK1/2 but higher phosphorylated ERK1/2 level in QH (25 and 50 μM) treated astrocytes in comparison to LPS alone was observed after 1 hr LPS incubation (Fig.

5.3). ERK regulates IL-1β, IL-6, TNF-α, MCP-1, iNOS transcription (Font-Nieves et al. 2012). Thus, increased pERK1/2 level in QH treated LPS induced astrocytes could be responsible for the weak lowering effect on cytokines expressions in QH (25μM) treated cells.

IκB-α is bound to NF-κB in normal condition and LPS induces phosphorylation of IκB-α that causes detachment of NF-κB which leads to up regulation of cytokine production. Reduced p-IκB-α protein level in QH (50, 100 or 200) groups whereas increased level in QH (25μM) group were observed (Fig. 5.4).TNF-α and COX-2 are the genes under the control of NF-κB pathway in astrocytes (Gorina et al. 2011, Font-Nieves et al. 2012, Lu et al. 2010) which were down regulated in the QH (50, 100 or 200μM) doses but not in QH (25 μM) dose treated cells. It has been reported that the nuclear p38-dependent acetyl transferase activity is essential for the LPS-induced NF-κB transcription activity in astrocytes (Gorina et al. 2011). Interestingly, the quite similar pattern of phospho p38 and p-IκB-α expression were observed.

5.5 DHA down-regulates IL-1β, COX-2 and TLR-4 mRNA levels in LPS activated astrocytes and TNF-α mRNA level in C6 cell line

To evaluate effect of DHA on gene expression of pro-inflammatory markers, DHA (25μM, 100μM) was incubated for 12 hrs followed by 6 hrs LPS incubation. In LPS treated astrocytes, DHA (25μM & 100μM) showed reduced IL-1β cytokine gene expression (Fig.5.5). It did not affect IL-6 & TNF-α genes expression. Further, HO-1 mRNA was observed to be substantially increased whereas; TLR-4 gene expression was decreased in 100μM DHA treated LPS induced astrocytes. TNF-α gene expression was significantly reduced in DHA (25μM & 100μM) treated LPS induced C6 glial cells.

There are no reports till date focusing on DHA's effect on gene expression of pro-inflammatory markers in astrocytes but IL-6 and TNF-α cytokines release was lowered in palmitic acid induced astrocytes (Gupta et al. 2012). Pre-incubation with DHA (100 μM) decreased IL-1β, IL-6 and TNF-α mRNA expression and DHA (25 μM) showed reduced IL-1β and IL-6 production in LPS stimulated macrophage (Gorjão et al. 2009). Furthermore, DHA has been observed reducing IL-6 production in lymphocytes (Khalfoun et al. 1997); inhibiting IL-2, IL-10 and IFN-γ production by Jurkat cells (human T lymphocytes) (Verlengia, Gorjão, Kanunfre, Bordin, De Lima, Martins, and Curi 2004); decreasing TNF-α, IL-10 and IFN-γ production by Raji cells (human B lymphocytes) (Verlengia, Gorjão, Kanunfre, Bordin, de Lima, Martins, Newsholme, et al. 2004); inhibiting IL-1βand TNFα production by monocytes (Chu et al. 1999); and suppressing

IL-6 and IL-8 generation by endothelial cells (De Caterina et al. 1994, Khalfoun et al. 1997). In various animal studies also DHA administration in fish oil has shown inhibiting effect on IL-1β, IL-6 and TNF-α mRNA levels which confirms DHA's role in regulating gene expression and thus inflammation (Anderson and Ma 2009, Robinson et al. 1996, Renier et al. 1993, Chandrasekar and Fernandes 1994). Clinical studies with DHA treatment also show reduced IL-1β, IL-2, IL-8, IL-10, or TNFα levels in disease condition (Kelley et al. 2009, Mori et al. 2003, Mozaffarian and Wu 2012).

5.6 DHA modulates pERK1/2 protein level in LPS treated astrocytes

Phosphorylated ERK1/2 protein expression was determined by western blot analysis after 30min and 6 hrs LPS incubation. As discussed previously, ERK regulates IL-1β, IL-6, TNF-α, MCP-1, iNOS transcription in astrocytes (Font-Nieves et al. 2012) and increased activation of the ERK1/2 further leads to increased cytokines release (Gupta et al. 2012). In the present study, the pERK1/2 expression was found to be increased at 30min LPS exposure and back to basal level in all the groups at 6 hrs LPS incubation. However, in 30min LPS incubation, pERK1/2 level was found to be more increased in DHA (100 μM) treated astrocytes compared to LPS alone (Fig.5.6).

Previous study has demonstrated that DHA (25μM) induced release of growth factor GDNF in astrocytes is mediated by ERK activation (Zhang et al. 2017).Another study also show higher pERK1/2 level in DHA (100 μM) treated neurons (Wellhauser and Belsham 2014) and suggest that anti-inflammatory effect of DHA is ERK-pathway independent.

Fig.5.1 Time dependent effect of LPS on gene expression of inflammatory markers Astrocytes were incubated with LPS (1 μg/ml) for 30 min, 4 hrs, 8 hrs, 12 hrs and 24 hrs. The mRNA levels were assessed by semi-quantitative RT-PCR. (a-f) Graphs for IL-1β, IL-6, TNF-α, COX-2, HO-1 and TLR-4 gene expression normalized to GAPDH and compared with control. Data are presented as mean ± SEM (n=4-6) and are from six independent experiments (*P < 0.05, **P < 0.01 ***P < 0.001, ****P < 0.0001)

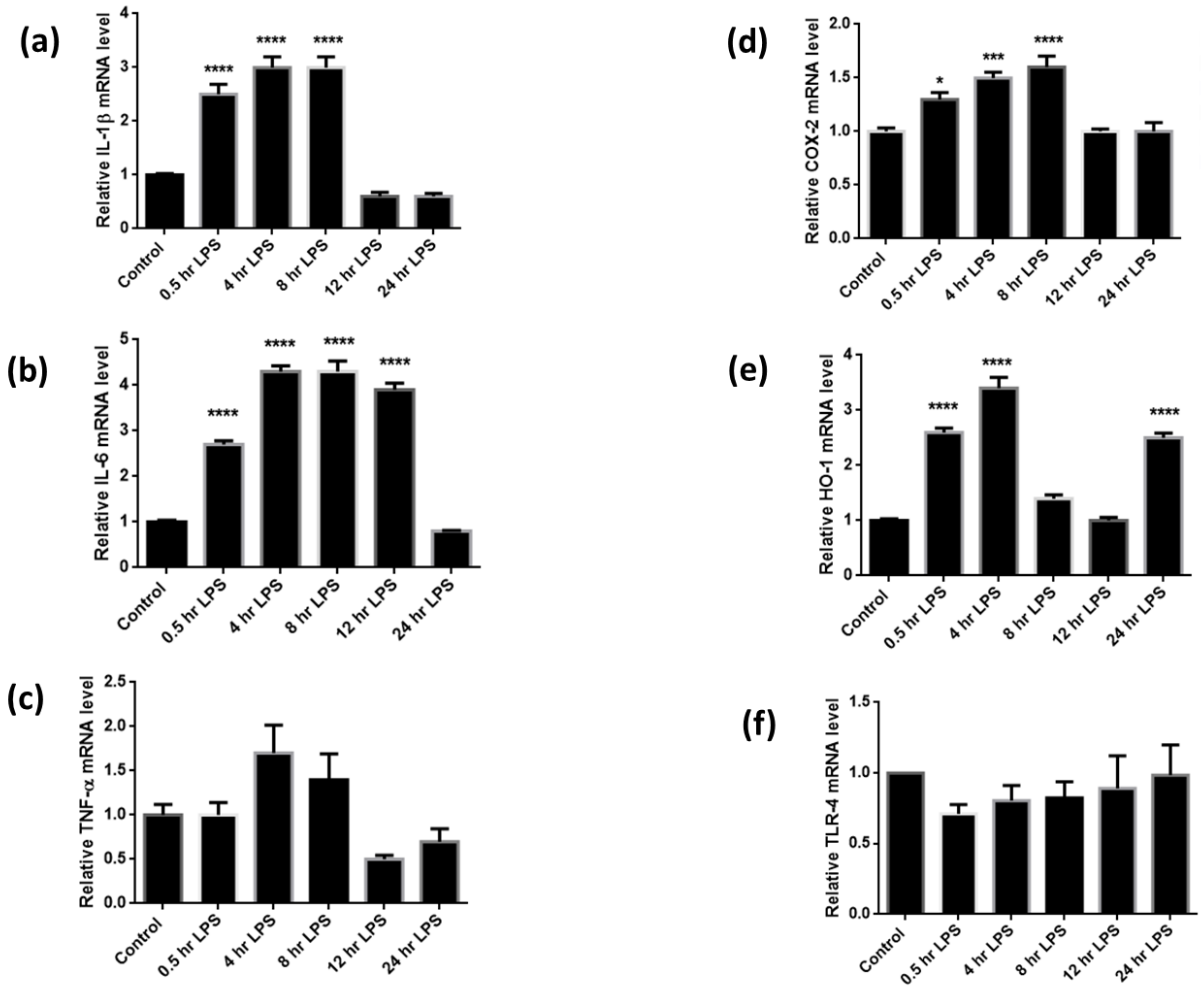


Fig.5.2 Quercetin reduces gene expression of inflammatory markers in a dose dependent manner Astrocytes were pre-incubated for 2 hrs with QH concentrations (25, 50, 100, 200 μM) followed by LPS (8 hrs ,1 μg/ml). The mRNA levels were assessed by semi-quantitative RT-PCR.(a-f) Graphs for IL-1β, IL-6, TNF-α, COX-2, HO-1 and TLR-4 genes expression normalized to GAPDH and expressed as relative to control. Data are presented as mean ± SEM (n=4-6) and are from six independent experiments. Statistical difference between QH treated groups with LPS alone were determined by one-way ANOVA(*P < 0.05, **P < 0.01 ***P < 0.001 , ****P < 0.0001)

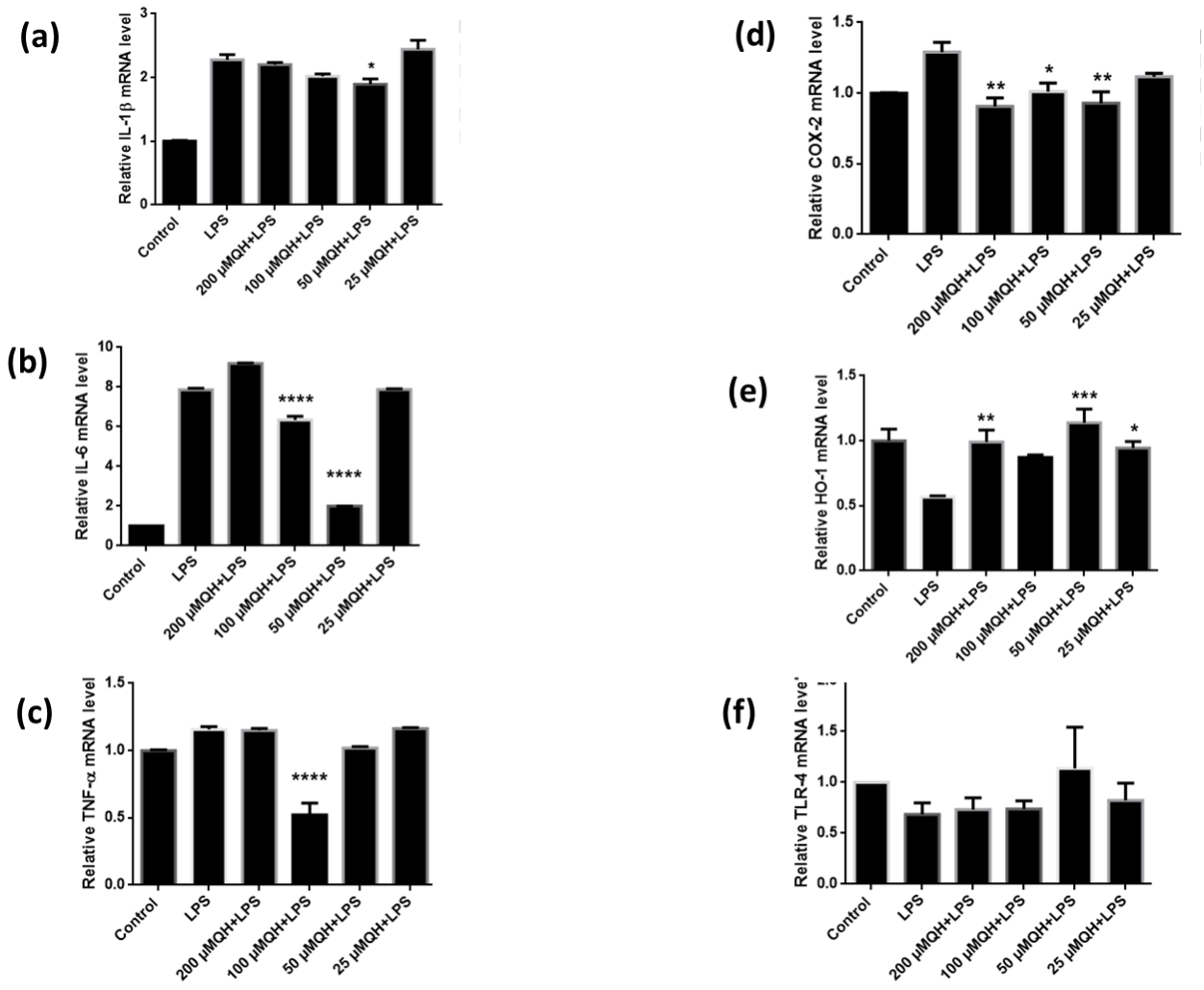


Fig. 5.3 Effect of quercetin on MAP kinase pathway and Glial fibrillary acidic protein (GFAP) Astrocytes were treated with various concentrations of QH for 2 hrs followed by LPS (1hr, 5 µg/ml) stimulation. ERK1/2 and p38 phosphorylation were examined by western blotting (a) Representative images for phospho p38, pERK1/2, GFAP and β-actin. (b-d) Graphs for proteins levels normalized to β-actin. Data represented as mean + SEM (n=3-4) from four independent experiments Statistical difference between QH treated groups with LPS alone were determined by one-way ANOVA(*P < 0.05, **P < 0.01 ***P < 0.001 , ****P < 0.0001)

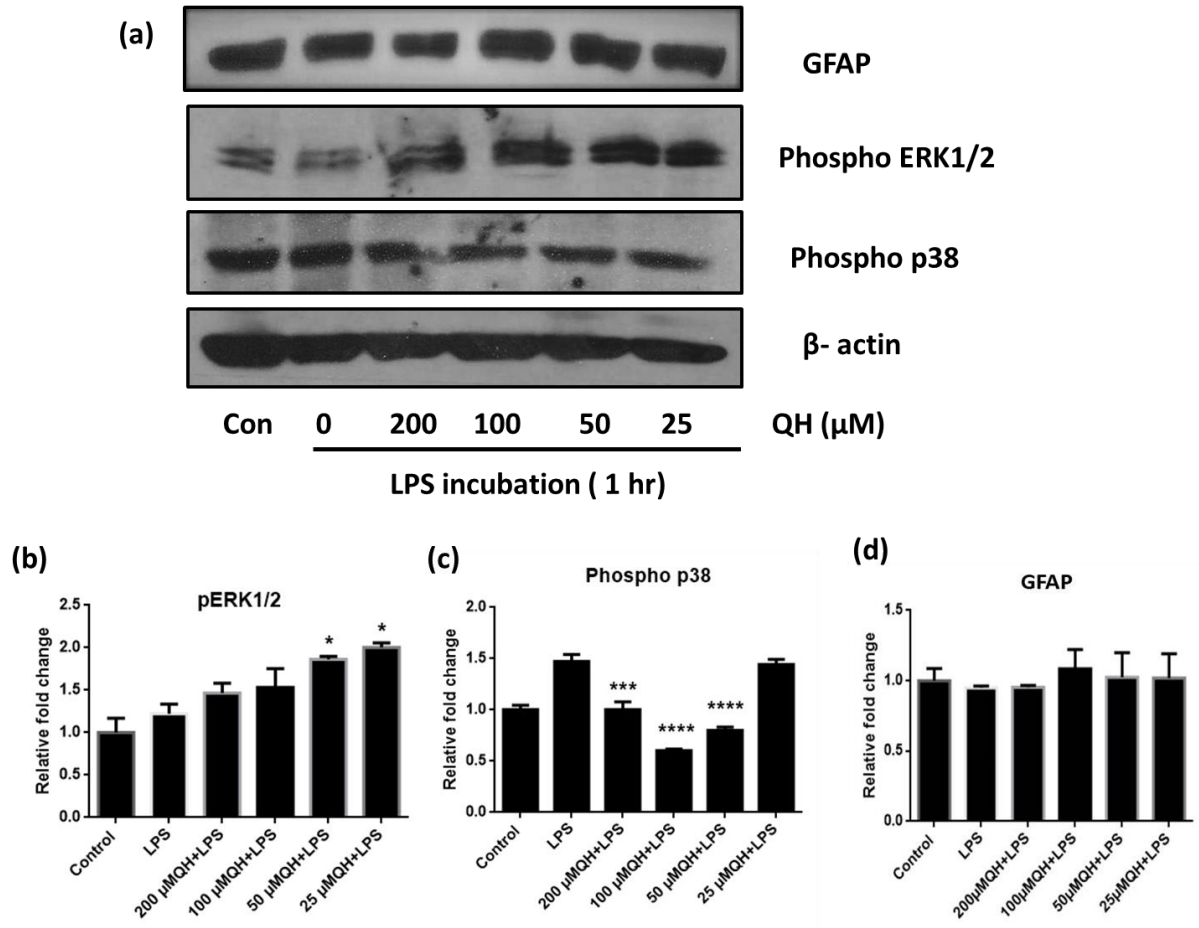


Fig. 5.4 Effect of quercetin on NF-κB pathway Astrocytes were treated with various concentrations of QH for 2 hrs followed by LPS (1hr, 5 μg/ml) stimulation. IκB-α phosphorylation was examined by western blotting (a) Representative images for p-IκB-α and β-actin. (b) Graph for proteins levels normalized to β-actin. Data represented as mean ± SEM from (n=3) from four independent experiments Statistical difference between QH treated groups with LPS alone were determined by one-way ANOVA(*P < 0.05, **P < 0.01 ***P < 0.001 , ****P < 0.0001)

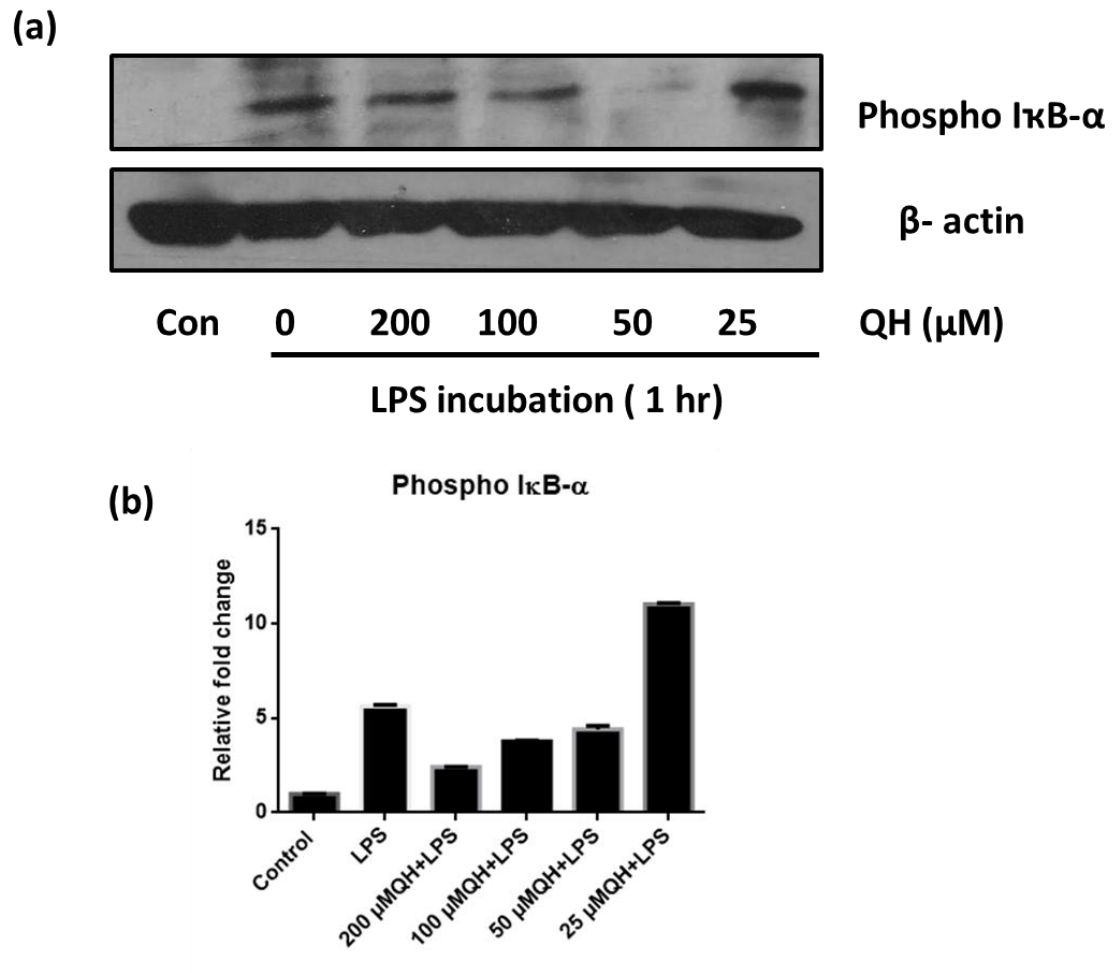
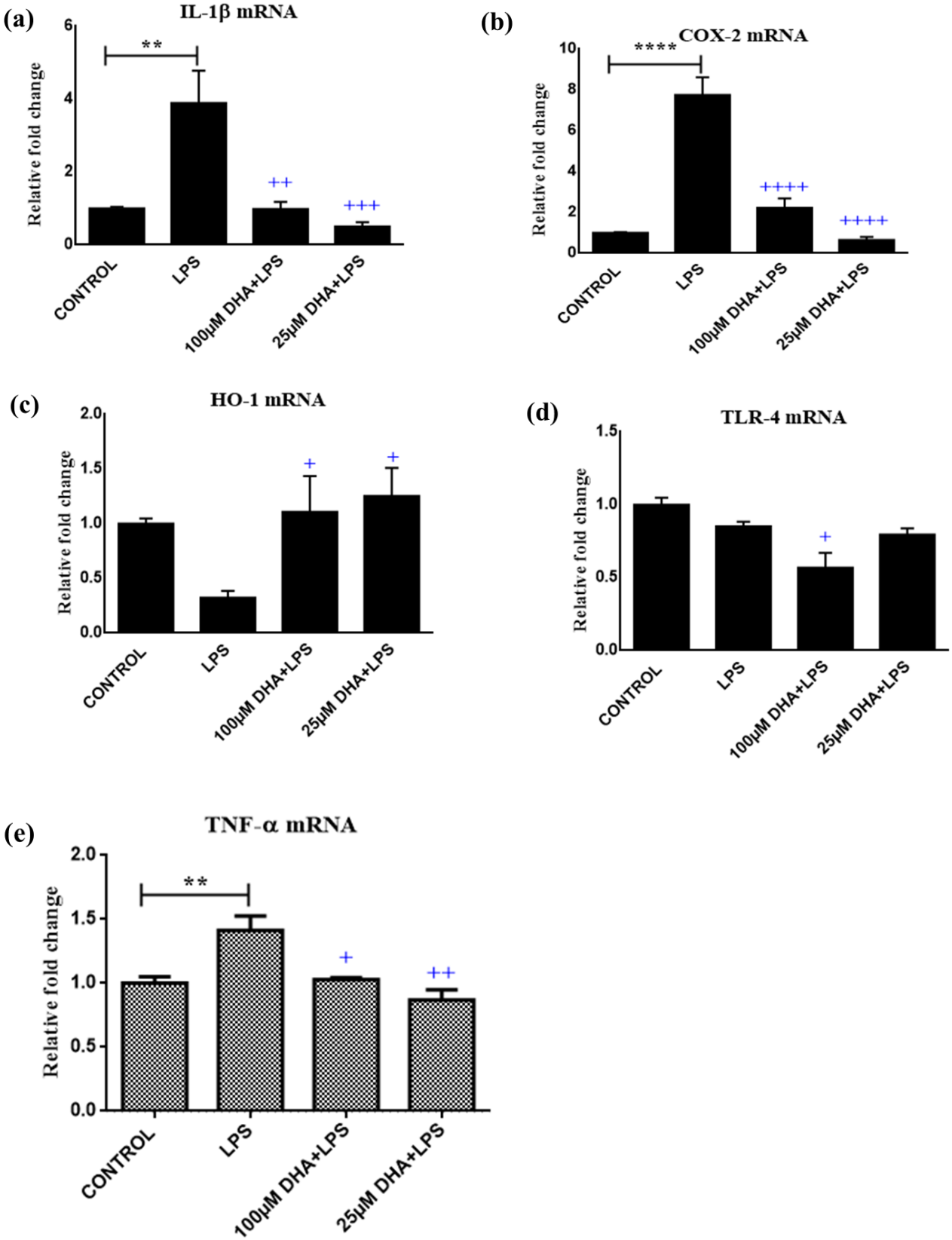


Fig. 5.5 DHA reduces gene expression of inflammatory markers in astrocytes and C6 glial cells Astrocytes and C6 glial cells were pre-incubated for 12 hrs with DHA concentrations (25, 100 μM) followed by LPS (6 hrs ,5 μg/ml). The mRNA levels were assessed by semi-quantitative RT-PCR. (a-d) Graphs for IL-1β, COX-2, HO-1 and TLR-4 genes expression in astrocytes normalized to GAPDH and expressed as relative to control.(e) Graph for TNF-α gene expression in C6 glial cells normalized to GAPDH and expressed as relative to control. Data are presented as mean ± SEM (n=3-4). Statistical difference between DHA treated groups with Control is marked with asterisks (**P < 0.01, ****P < 0.0001) and with LPS alone is marked with plus sign (+P < 0.05, ++P < 0.01 +++P < 0.001, ++++P < 0.0001).


(a)
IL-1β mRNA
Relative fold change
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CONTROL
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100μM DHA+LPS
25μM DHA+LPS
(b)
COX-2 mRNA
Relative fold change
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LPS
100μM DHA+LPS
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(c)
HO-1 mRNA
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25μM DHA+LPS
(d)
TLR-4 mRNA
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(e)
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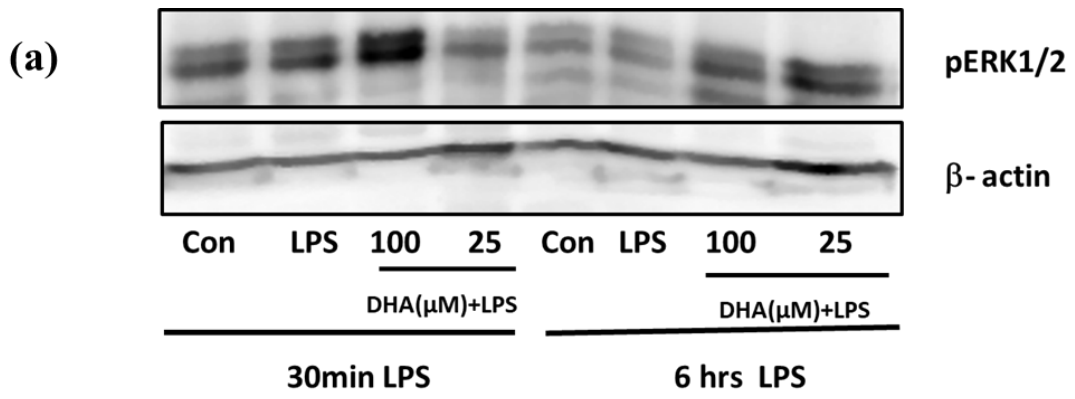
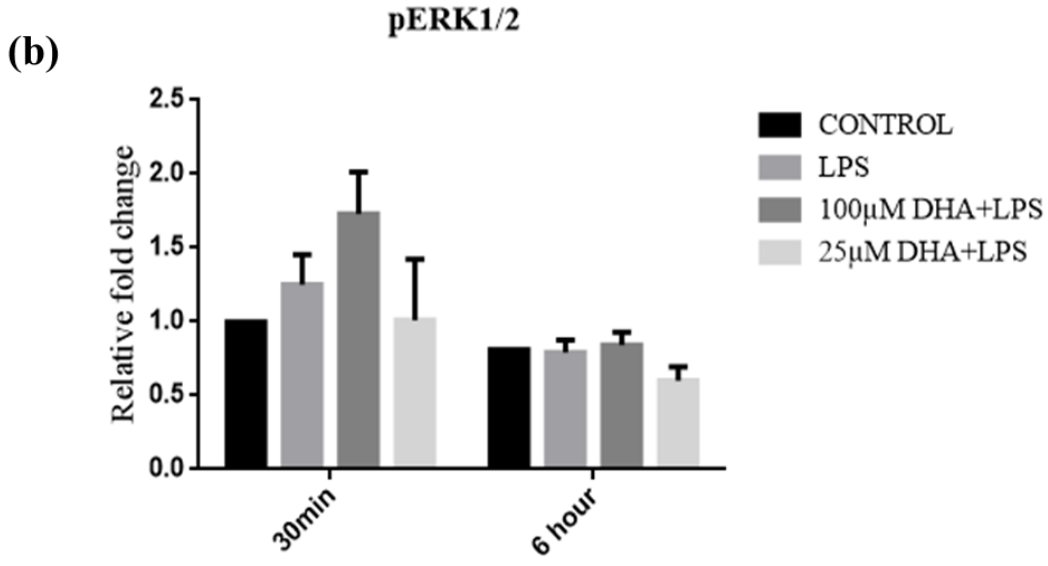
100μM DHA+LPS
25μM DHA+LPS

Fig. 5.6 DHA modulates pERK1/2 protein expression in LPS activated astrocytes Astrocytes were treated with DHA for 12 hrs followed by LPS (30min & 6 hrs, 5 µg/ml) stimulation. pERK1/2 expression was examined by western blotting (a) Representative image for pERK1/2 and β-actin. (b) Graph for protein level normalized to β-actin. Data represented as mean ± SEM (n=3).Statistical difference between DHA treated groups with LPS alone were determined by one-way ANOVA





5.4 Summary

In the present study, LPS alone did not induce ligand dependent TLR-4 mRNA level alteration in astrocytes. 1μg/ml LPS causes significant up regulation of pro-inflammatory molecules-IL-1β, IL-6, TNF-α and COX-2 in a time dependent manner. LPS incubation for 8 hrs was found to be the optimal time point to observe the elevated gene expression of above mentioned pro-inflammatory markers. Data show that quercetin hydrate (100μM and 50μM) down regulates the IL-1β, IL-6, TNF-α, COX-2 and up regulates HO-1 genes expression in a dose dependent manner. Signalling molecules involved in regulation of cytokines production were checked by assessing their phosphorylated forms (phospho p38, p-IκB-α and pERK1/2) as their alteration is ultimately responsible for transcription of inflammatory molecules. These signalling molecules were found to be modulated which were concomitant with the gene expression of inflammatory markers and in line with earlier reports. Surprisingly, 200μM QH significantly decreased phospho p38 and p-IκB-α proteins but did not lower cytokines mRNA levels that is unexplainable and has to be investigated further. Taken together, the study suggests that quercetin possess potential to down regulate pro-inflammatory markers and up regulate anti-inflammatory marker transcription by altering activation of signalling pathways. These mRNA levels changes might enable the lowering of the pro-inflammatory cytokines release ultimately. Furthering our study of quercetin on the protein levels and release of all the cytokines will provide the complete understanding of its anti-inflammatory effects in astrocytes to employ it for further research in line for its therapeutic use in astrocytic inflammation mediated brain diseases.

DHA treatment reduced IL-1β, COX-2 and TLR-4 mRNA levels and increased HO-1 mRNA level; the other cytokines IL-6 & TNF-α were unaltered in LPS activated astrocytes. pERK1/2 level was more increased after 30min LPS exposure in DHA pre-incubated astrocytes as compared to LPS alone. As the existing results show inconsistencies in cytokines expression further investigation is needed using other DHA concentrations to conclude its effect in reactive astrocytes.