Modulation of BV-2 microglia functions by novel quercetin pivaloyl ester

Nataša Mrvová a, Martin Škandík a, Marcela Kuniaková b, Lucia Račková a,∗

a Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences, Dubravská cesta 9, 841 04 Bratislava, Slovak Republic
b Faculty of Medicine Institute of Medical Biology, Genetics and Clinical Genetics, Faculty of Medicine, Comenius University, Sasinkova 4, 811 08 Bratislava, Slovak Republic

A R T I C L E   I N F O

Article history:
Received 7 May 2015
Received in revised form 28 August 2015
Accepted 14 September 2015
Available online 16 September 2015

Keywords:
BV-2 microglia
Anti-inflammatory efficacy
Quercetin
3’-O-[(3-chloropivaloyl)quercetin

A B S T R A C T

Chronic inflammation in brain plays a critical role in major neurodegenerative diseases such as Alzheimer’s, Parkinson’s disease, stroke or multiple sclerosis. Microglia, resident macrophages and intrinsic components of CNS, appear to be main effectors in this pathological process. Quercetin, a naturally occurring flavonoid, was proven to downregulate inflammatory genes in microglia. Synthetically modified quercetin, 3’-O-[(3-chloropivaloyl)quercetin (CPQ), is assumed to possess better biological availability and enhanced antioxidant properties.

In the present study, antineuroinflammatory capability of the novel compound CPQ was assessed in BV-2 microglial cells. Our data show that treatment with CPQ attenuated the production of the inflammatory mediators, nitric oxide (NO) and tumour necrosis factor-α (TNF-α), in LPS-stimulated microglia somewhat more efficiently than did quercetin (p < 0.05 for CPQ vs. quercetin-treated group). Also, protein level of inducible NO synthase (iNOS) in LPS-activated BV-2 microglia was to some extent more effectively suppressed by CPQ than by unmodified flavonoid. In consistence with the extent of their effects on pro-inflammatory markers, CPQ and quercetin showed down-regulation of NFκB activation. This quercetin analogue caused also a decline in BV-2 microglia proliferation with some extent more effectively supressed by CPQ than by unmodified flavonoid. These results point to the therapeutic potential of 3’-O-[(3-chloropivaloyl)quercetin (CPQ) as a novel anti-inflammatory drug in neurodegenerative diseases, mediating favourable modulation of pro-inflammatory functions of microglia.

© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

Microglia are resident macrophages and main immunocompetent cells of brain. In healthy conditions, they are characterized by resting phenotype and they are scanning their environment (Olah et al., 2011). Initial engagement of microglia seems to be neuroprotective. Microglia respond to and propagate inflammatory signals following activation. These activated microglia become highly motile, secreting inflammatory cytokines and chemokines, migrating to the lesion area, and phagocytosing cell debris or damaged neurons (Fu et al., 2014).

However, the prolonged inflammatory response of activated microglia contributes to changes in physiological processes in the CNS and leads to neuronal damage (Cunningham, 2013; Eufemi et al., 2015). Ageing can result in the pathogenic alteration of...
microglial functions leading to persistent microglial over-activation, failure to respond correctly to stimuli and subsequent eventual neurodegeneration. Over-activated microglia produce diverse neurotoxic mediators such as pro-inflammatory cytokines, nitric oxide (NO), reactive oxygen species (ROS) (Suk et al., 2003). Chronic microglial activation and neuroinflammation is closely associated with pathogenesis of several neurodegenerative diseases including Alzheimer’s disease (AD), Parkinson’s disease (PD), Huntington’s disease (HD), multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS) (Choi et al., 2011). Thus, inhibition of microglia activation and interference with neuroinflammatory processes may provide promising therapeutic strategy against development and progression of neurodegenerative diseases.

Over 6000 different flavonoids have been identified and they represent the most common, widely distributed group of plant phenolics. Flavonoids are widely distributed in fruit, vegetables, nuts, grains, and tea. A number of epidemiological studies observed variety of clinically relevant properties such as their antioxidant, antibacterial, antiviral, antimutagenic, anticancer, anti-inflammatory properties, their interactions with intracellular signalling pathways, the regulation of cell survival/apoptotic genes and mitochondrial function. In addition, there is a dose-dependent association between intake of these substances in food and cognitive performance (Choi et al., 2012a; Jang and Johnson, 2010; Spencer et al., 2012). Recently, there has been much interest in the neuroprotective effects of flavonoids, which were shown in effective protection of both cognitive and motor functions against ageing-related decline in animal models and human studies (Spencer et al., 2012). Jang and Johnson (2010) suggested that flavonoids in a diet might positively modulate the functions of ageing microglia and possibly revert their senescence-related changes. Naturally occurring flavonols such as quercetin, were also suggested to beneficially influence microglia in neuropathologies. In vitro, quercetin inhibited microglia activation and protected neurons against damage (Kao et al., 2010). Moreover, the microglial inhibitory pathway was reported to be involved in the suppression of neuroinflammation-apoptotic cascade by quercetin in the rat model of depression (Rinwa and Kumar, 2013).

Studies using in vitro models mimicking aspects of the blood–brain-barrier confirmed that certain flavonoids can cross the brain endothelium (Youdim et al., 2003). Ability of flavonoids to cross the blood–brain barrier in vivo was also reported and the permeability was suggested to be influenced by their lipophilicity and interactions with efflux transports (de Boer et al., 2005; Youdim et al., 2004a,b). Chemical synthesis might represent an efficient tool for rational modification of flavonoid structure and thus optimization of biological availability and effects of these natural substances.

In the presented study, the novel synthetically modified flavonoid, 3’-O-(3-chloropivaloyl)quercetin (CPQ), with optimized physico-chemical properties was evaluated in view of its efficacy to modulate pro-inflammatory functions of microglia, using the model of BV-2 microglial cell line.

2. Material and methods

2.1. Materials

All reagents were of analytical grade or the highest possible purity. Rabbit polyclonal antibody to iNOS, rabbit polyclonal antibody to β-actin and rabbit polyclonal antibody to NF-κB p65 were purchased from Cell Signalling Technology Inc. (Biotech, s. r. o., Bratislava, Slovakia). Rabbit polyclonal antibody to arginase 1 was from Santa Cruz Biochemicals, Inc. Rabbit polyclonal antibody to Histone H2A.X was obtained from Abcam (Cambridge, UK). Triton X-100 was from VWR International, s.r.o. (Bratislava, Slovakia), Hepses from AppliChem (Labo - sk, s.r.o., Bratislava, Slovakia).

Thiazoyl blue tetrazolium bromide (MTT), nitroblue tetrazolium (NBT), lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 (L4391), phorbol 12-myristate 13-acetate (PMA), HRP-linked IgG anti-rabbit antibody, quercetin, sodium dodecyl sulphate (SDS), albumin from bovine serum (BSA), phenylmethysulfonyl fluoride (PMSF), propidium iodide (PI), ribonuclease A from bovine pancreas (RNase), sucrose, Trypan Blue and other chemicals were obtained from Sigma–Aldrich (Bratislava, Slovakia), unless otherwise stated.

The compound CPQ (99.5% purity, HPLC), was synthetized by Veverka et al. (2013) and the structure of the compounds tested is outlined in Fig. 1.

2.2. Cell culture and administration of the compounds tested

The immortalized mouse microglial cell line BV-2 was developed in the laboratory of Dr. Blasi at the University of Perugia (Blasi et al., 1990). The cells were cultured in DMEM (Sigma–Aldrich, Bratislava, Slovakia), supplemented with 10% FBS (PAA, Biotech, s. r. o., Bratislava, Slovakia), and 1% P/S (100 U/ml penicillin, 100 μg/ml streptomycin, K-Trade, s.r.o., Bratislava, Slovakia) and maintained in 5% CO₂ at 37 °C. Cells were used for 10 passages at maximum.

The stock solutions of substances tested were prepared in DMSO. The final concentration of the vehicle in DMEM was 0.35% in all the samples tested. The stock solution of LPS (1 mg/ml) was prepared in Hank’s balanced salt solution (PAA, Biotech, s. r. o., Bratislava, Slovakia). The inflammmogen was added to the cells in the presence of the compounds tested or a vehicle.

2.3. Direct cell counting

BV-2 cells were seeded in 24-well plates. After 24 h exposure to the compounds tested cells were harvested, washed twice with phosphate-buffered saline (PBS), stained with Trypan blue (0.2% in PBS) and counted in counting chamber.

Percentage of the cell population growth or relative cell proliferation inhibition was calculated by the formula:

\[
\% \text{ of growth} = \left\{ \frac{(Exp - C_0)}{(C_d - C0)} \right\} \times 100\%
\]

where Exp is the number of cells after 24 h of cultivation with the compounds tested, C₀ is the number of cells at the time of treatment and C_d is the number of cells after 24 h of cultivation with the vehicle.

2.4. MTT assay

The cells were grown in 96-well microplates, in complete DMEM. At the end of the incubation with or without the substances
tested, the cells were incubated with MTT (0.5 mg/ml) in DMEM in 5% CO₂ at 37 °C for 120 min. Subsequently, 100 μl of 10% SDS in HCl (0.01 M) was added and the cells were thoroughly resuspended. The absorbance was spectrophotometrically recorded at 570 nm using the reference (690 nm).

2.5. Cell cycle analysis

BV-2 cells were plated in 6-well dishes and allowed to attach overnight. After treatment with the compounds tested, the cells were washed with PBS and trypsinized. Next, the cells were pelleted and adjusted to a concentration of 1–2 × 10⁶ cells/ml and fixed by adding 50–150 μl of cell suspension to 70% ethanol drop-wise with continuous mixing. After fixation overnight at −20 °C, the cells were pelleted and resuspended gently in ice-cold PBS. After 2 washes, cells were suspended in propidium iodide (PI) staining solution (10 μg/ml PI, 100 μg/ml RNase, 0.1% (v/v) Triton-X) for 15 min at 37 °C in the dark. The cell cycle was analysed using a flow cytometer (Beckman Coulter FC500). Data were analysed using MultiCycle AV software and a minimum of 5000 cells per sample were analysed.

2.6. Production of NO by BV-2 microglia

BV-2 cells were plated in 96-well dishes and allowed to attach overnight. Then, they were treated with LPS and/or the compounds tested for 16 h. Aliquots of conditioned media were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylenediamine in 2.5% phosphoric acid) in clean 96-well plates. Absorbance was measured at 540 nm with reference (690 nm) using a microplate reader. Nitrite concentrations were calculated using a sodium nitrate standard curve prepared in cell culture medium.

2.7. Oxidative burst assay

The respiratory/oxidative burst assay was conducted using the nitroblue tetrazolium (NBT) reduction method as described by (Rook et al., 1985). Briefly, BV-2 microglia were plated in 96-well dishes. After exposure of the cells to serum-free conditions for 16 h with or without LPS (1 μg/ml), cells were treated with 2 μmol/l PMA and/or the compounds tested for 1.5 h in the presence of NBT (0.75 mg/ml) in DMEM in 5% CO₂ at 37 °C. At the end of the incubation, the cells were washed twice with 70% methanol and the precipitate of reduced NBT was dissolved with mixture of KOH/ DMSO. The absorbance was recorded at 630 nm by using a microplate reader.

2.8. Production of TNF-α by BV-2 microglia

Production of TNF-α was measured according to manufacturer’s protocol: RayBio® Mouse TNF-alpha ELISA Kit (Baria S.r.o., Prague, Czech Republic). Briefly, BV-2 cells were seeded in 96-well plates and allowed to attach overnight. Then, they were treated with LPS and/or the compounds tested for 16 h. Aliquots of conditioned media were pipetted to TNF-α Microplate and incubated for 2.5 h, followed by incubation with biotin antibody, Streptavidin solution, TMB One-Step Substrate Reagent. Reaction was stopped by adding Stop Solution and absorbance was measured immediately at 450 nm. TNF-α concentrations were calculated by using a standard curve.

2.9. Western blot analysis

The cells were lysed in detergent lysis buffer containing protease inhibitors (Cell Signalling Technologies Inc., Biotech, s. r. o., Bratislava, Slovakia) with 1 μmol/l PMSF, then homogenized by passing 20 times through a 23G-needle, incubated on ice for 20 min, and subsequently cleared by centrifugation for 10 min at 14,000 rpm at 4 °C. Protein concentration in cell lysates was determined by using the Bradford assay (BioRad, s.r.o., Praha, Czech Republic). An equal amount of proteins for each sample was separated by 7.5% SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Santa Cruz Biotechnology, Inc., Heidelberg, Germany). The membrane was blocked by 3% BSA, and sequentially incubated with primary antibodies (iNOS dilution 1:700, Arginase-1 dilution 1:300) and horseradish peroxidase-conjugated secondary antibody (dilution 1:10,000). Blots were developed with a chemiluminescent system (Western Blotting Luminol Reagent, Santa Cruz Biotechnology, Inc.; according to the manufacturer’s instructions). Densitometric analyses of western blots were performed by using software ImageJ (National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/).

2.10. NFκB translocation analysis

BV-2 cells were plated in 6-well dishes and allowed to attach overnight. After treatment with the compounds tested, the cells were washed with PBS and trypsinized. Next, the cells were pelleted and adjusted to a concentration of 1–2 × 10⁶ cells/ml. Then, the cells were resuspended in nuclei extraction buffer (1% Triton X-100 (v/v), 10 mmol/l Hepes, 320 mmol/l sucrose, 5 mmol/l MgCl₂, pH 7.4), gently mixed and incubated for 10 min on ice. Isolated nuclei were washed 2 times with ice-cold wash buffer (10 mmol/l Hepes, 320 mmol/l sucrose, 5 mmol/l MgCl₂, pH 7.4), then lysed with lysis buffer containing protease inhibitors with PMSF and incubated for 10 min on ice. Protein concentration in nuclei lysates was determined using the Bradford assay. An equal amount of nuclei fraction for each sample was separated by 10% SDS polyacrylamide gel. Proteins were transferred to nitrocellulose membrane. The membrane was blocked using 3% BSA for 1 h at room temperature and then incubated with primary antibody: anti-NFκB p65 (dilution 1:1000) or anti-Histone H2A.X (dilution 1:2500) and horseradish peroxidase-conjugated secondary antibody. The blots were developed using a chemiluminescent detection (Western Blotting Luminol Reagent) and densitometric analyses of western blots were performed using software ImageJ.

2.11. Phagocytosis assay

The phagocytic activity of microglial cells was determined by using fluorescent yellow-green carboxylate-modified polystyrene latex beads (1 μm; Sigma, Bratislava, Slovakia) and FITC-labelled S. aureus (Kronek et al., 2010). A suspension of beads (final concentration 0.05% v/v) was added to the culture medium 16 h after addition of LPS (10 μg/ml) in the presence or the absence of the compounds tested and was allowed to incubate for 180 min. Alternatively, a suspension of bacteria (dilution 1:2000) was added for 180 min to the cells incubated for 16 h with or without the compounds tested. Cells were rinsed three times in PBS to remove nonadherent particles and were collected by trypsinisation. Afterwards, the pelleted cells were fixed in 2% formaldehyde/0.2% glutaraldehyde in PBS, washed twice with PBS and fluorescence was evaluated by using a flow cytometer (Beckman Coulter FC500). For each sample, the fluorescence histogram of 5000 cells was generated and analysed. Gates were set around debris and intact cells on forward scatter vs. side scatter dot plot. Data were analysed by using CXP software (Beckman Coulter). Two types of parameters were calculated from the obtained histogram. Firstly, the phagocytic rate, which represents the percentage of cells with
2.12. Statistical analysis

Results are expressed as means ± standard deviation (SD) from at least three separate experiments run at least in duplicates. The replicates were processed all on the same day, the independent experiments were run on different consecutive days. Prior to testing for mean effects, data were tested for normality of distributions using Shapiro–Wilk test and Levene’s test of equality of variances between compared groups was used. For multiple comparisons, values of p were calculated using one-way ANOVA with Tukey’s post-hoc analysis (Fig. 4A) or Dunnett’s post-hoc analysis (Figs. 2A and B; 3A and B) where homogeneity of variance was met. Otherwise, Welch’s robust test of equality of means was used followed by Games–Howell post-hoc analysis (Figs. 4A and B; 5C and D; 6B) or Dunnett’s T3 post-hoc analysis (Fig. 3C). SPSS software (Version 22.0) was used. Statistical significance is expressed as *p < 0.05, **p < 0.01 and ***p < 0.001 vs. unstimulated control or #p < 0.05, ##p < 0.01 and ###p < 0.001 vs. stimulated cells. Statistically significant differences concerning CPQ vs. quercetin groups are expressed as *p < 0.05, **p < 0.01 and ***p < 0.001. In the Figs. 4–6, statistical significance is shown only for treated cells vs. stimulated cells.

3. Results

3.1. Cytotoxicity of CPQ and quercetin

Twenty-four-hours pre-treatment with CPQ and quercetin (Fig. 2B) lead to diminution of MTT conversion at higher concentrations tested. Remarkably greater influence of MTT reduction was observed in the cells treated with CPQ than with quercetin (74.6 ± 6.9% of control, p < 0.01, compared to 96.8 ± 7.1% of control, respectively, at concentration 35 μmol/l; p < 0.01 for CPQ vs. quercetin group). However, CPQ did not show any impact on viability of the cells, as confirmed by intact formazan development in MTT-exposed cells (Fig. 2C) as well as minimum uptake of trypan blue (data not shown) but the cell proliferation was influenced. This was further confirmed by the respective cell counts showing decreased percentage of growth for CPQ as well as quercetin at the highest concentration used (62.5 ± 3.7%, p < 0.001, and 90.4 ± 6.2%, p < 0.05 respectively; p < 0.05 for CPQ vs. quercetin group, Fig. 2A).

3.2. Influence of cell cycle by CPQ and quercetin

Flow cytometry analysis confirmed that CPQ as well as quercetin can influence the cell cycle profile of BV-2 cells. As shown in Fig. 3, both compounds tested caused significant decrease in the number of cells in G1 phase of resting BV-2 microglia with the larger effect of CPQ (52.2 ± 0.6%, p < 0.001, and Q 59.8 ± 0.8%, p < 0.001, at concentration 35 μmol/l; p < 0.01 for CPQ vs. quercetin group). Consequently, this was followed by a significant accumulation of the cells in G2/M phase (22.3 ± 0.7%, p < 0.001, and 16.4 ± 0.5%, p < 0.01, at concentration 35 μmol/l; p < 0.001 for CPQ vs. quercetin group). Thus, with regard also to the cytotoxicity data, CPQ can cause a notable decline in BV-2 microglia proliferation along with interference with cell cycle progression. In addition, CPQ-induced cell cycle arrest in G2/M phase had negligible impact on cell viability.

3.3. Modulation of NO release by CPQ and quercetin

Stimulation with LPS (10 μg/ml) induced a remarkable
production of NO (p < 0.001) (Fig. 4A). Both compounds tested caused a significant and concentration-dependent decrease in NO production with the more profound effect of CPQ (49.3 ± 15.2% of LPS-stimulated cells, p < 0.001 at concentration 10 μmol/l) compared to quercetin (75.8 ± 11.4%, p > 0.001 vs. LPS-stimulated cells; p < 0.001 for CPQ vs. quercetin group).

3.4. Effect of CPQ and quercetin on oxidative burst

Stimulation of BV-2 cells with PMA caused a significant accumulation of NBT-derived formazan (151 ± 18% of control, p < 0.05) pointing to superoxide burst (confirmed by its abolishment by application of superoxide dismutase (1 mg/ml, Fig. 4C)). This was notably potentiated by priming of the cells with LPS (1 μg/ml; 232 ± 40% of control, p < 0.001). CPQ at concentration 25 μmol/l showed mildly better suppression of NBT reduction than quercetin in both primed and non-primed cells (174 ± 15%, p < 0.05 and 190 ± 27% of control, in primed cells; 125 ± 25% and 139 ± 23% of control in non-primed cells for CPQ and quercetin, respectively). No inhibitory effect on oxidative burst of BV-2 cells was observed at the

---

**Fig. 3.** Inhibitory effect of the compounds tested on cell cycle progression of BV-2 cells. Cells were stained by propidium iodide and analysed by flow cytometry. (A) Representative histograms of the cell cycle of microglia incubated for 24 h with or without the compounds tested. Quantitative analysis of measured histograms: % of cells in G1 phase (B), S phase (C) and G2/M phase (D). Results represent means ± SD from 3 independent experiments run at least in duplicates, **p < 0.01, ***p < 0.001 vs. control. ”p < 0.01. ”p < 0.001 CPQ vs. Q; CON – control cells treated with 0.35% DMSO; CPQ – 3’−O-(3-chloropivaloyl) quercetin, Q – quercetin.

**Fig. 4.** Attenuation of inflammatory mediators and superoxide production by CPQ treatment in BV-2 cells. (A,B) Cells were stimulated by LPS (10 μg/ml), treated with the compounds tested for 24 h. Nitric oxide production (A) and TNF-α release (B) were measured in cell culture medium using Griess reagent and by ELISA, respectively. BV-2 cells for NBT test (C) were stimulated with 2 μmol/l PMA and co-treated with Q and CPQ (25 μmol/l) for 1.5 h following 16 h priming with/without LPS (1 μg/ml). (D) Light microscopy of production of NBT-derived formazan by PMA-stimulated BV-2 cells and effect of SOD compared to CPQ. Results represent means ± SD from 3 to 12 independent experiments run at least in triplicates, *p < 0.05, **p<0.01, ***p < 0.001 vs. control, ”p < 0.05, ”p < 0.01, ””p < 0.001 vs. stimulated cells and ”p < 0.05 CPQ vs. Q; CON – control cells treated with 0.35% DMSO; CPQ – 3’−O-(3-chloropivaloyl) quercetin, Q – quercetin, PMA – phorbol-12-myristate-13-acetate; LPS – lipopolysaccharide–stimulated cells; NBT – nitro blue tetrazolium; SOD – superoxide dismutase.
concentration 10 μmol/l (data not shown).

3.5. Modulation of TNF-α production by CPQ and quercetin

As shown in Fig. 4B, the production of TNF-α by BV-2 microglia was significantly induced in the LPS-stimulated cells (594 ± 49 pg/ml vs. 88 ± 1 pg/ml for control cells, p < 0.01).

The increased levels of TNF-α were significantly attenuated by both compounds tested with somewhat more remarkable effect of CPQ (44.4 ± 9.4% of LPS-stimulated cells, p < 0.05 at concentration 25 μmol/l) compared to quercetin (56.9 ± 12.0%; p > 0.05 for CPQ vs. quercetin group).

3.6. Influence of iNOS induction by CPQ and quercetin

LPS-stimulation elicited a dramatic increase in iNOS level (relative band intensity 0.97 A.U., vs. 0.07 A.U. for control, calculated from representative blot). Analogously to the modulation of other inflammatory markers, unlike quercetin, CPQ significantly repressed protein expression of iNOS (in the concentration range 1–10 μmol/l; Fig. 5A). Significant downregulatory effect of CPQ on iNOS levels was observed starting from 10 μmol/l concentration (0.25 ± 0.12 fold change of LPS-stimulated cells, p < 0.001). Hence, inhibitory effect of CPQ on NO production by activated microglia can be associated with a decrease of iNOS expression.

3.7. Effect of CPQ and quercetin on induction of NFκB translocation

We examined the effect of the compounds tested on LPS-induced nuclear translocation of NFκB, the transcription factor regulating numerous inflammatory genes (Khasnavis et al., 2012). Western blotting analysis of the nuclear protein extracts isolated from the LPS-stimulated cells showed the protein bands specific for NFκB/p65 (Fig. 5B), confirming thus its nuclear translocation and activation. In line with their effects on the inflammatory markers, both compounds tested showed quite comparable suppression of NFκB/p65 activation, however, with a significant effect of CPQ (relative band intensity for CPQ: 0.24 ± 0.12 A.U., p < 0.05, at 10 μmol/l, Fig. 5B).

Fig. 6. Influence of CPQ and quercetin on phagocytic activity of BV-2 cells. (A) At the top: representative histogram plots show microglia incubated for 3 h with latex beads following 16 h stimulation with/without LPS and at the bottom: for 3 h with FITC-labelled S. aureus following 16 h incubation, in the presence or the absence of 25 μmol/l CPQ. Percentage in the histograms (phagocytosis rate) represents number of microglial cells positive for latex beads/FITC-labelled bacteria relative to the total number of cells. The quantitative assessment of histograms for both compounds tested is shown on graph B. Phagocytosis index denotes the mean of fluorescence corresponding to the relative mean number of particles ingested per cell. Results represent means ± SD from 3 independent experiments run in duplicates, *p < 0.05, **p < 0.001 vs. control, CON — control cells treated with 0.35% DMSO; LPS — lipopolysaccharide-stimulated cells, CPQ — 3′-O-(3-chloropivaloyl) quercetin, Q — quercetin, both compounds tested in 25 μmol/l concentration.
3.8. Effect of CPQ and quercetin on M1/M2 microglial polarization markers

Microglia (comparably to peripheral macrophages) can adopt either a classically activated M1 phenotype or an alternatively activated anti-inflammatory M2 phenotype (Kennedy et al., 2013). Polyphenolic compounds, such as curcumin, were shown to promote switching of microglia from neurotoxic to neuroprotective phenotype (Lin, 2007). In consistence with data reported for BV-2 cell models (Michaud et al., 2013), LPS stimulation induced protein expression of M1 phenotype markers (iNOS and TNF-α) along with downregulation of arginase 1 (Arg-1), a sensitive marker of alternative macrophage activation (Fig. 5C and F). Treatment of LPS-stimulated BV-2 cells with 25 μM CPQ and quercetin led to a profound reduction of iNOS followed by an increase of Arg-1 protein levels. However, negligible differences for Arg-1 levels were seen between unstimulated controls and flavonoid-treated stimulated cells.

3.9. Effect of CPQ and quercetin on phagocytosis

The flow cytometry analysis was used to evaluate the effect of CPQ and quercetin on phagocytic activity of BV-2 microglia. A slight increase of phagocytosis rate (PhR) of latex beads (16.2 ± 0.1% for CPQ, compared to control 13.5 ± 0.2%) without affecting the phagocytosis index (PhI), was observed in the resting cells (CPQ: 10.5 ± 8.4 MFI, compared to control 9.6 ± 4.0 MFI, Fig. 6B). Stimulation with LPS markedly enhanced phagocytosis of latex particles (shown as significantly increased PhR and mildly elevated PhI, 38.9 ± 6.2%, p < 0.001 and 13.0 ± 6.2 MFI, p > 0.05). However, neither PhR nor PhI were affected by any of the compounds tested in comparison with the stimulated control (in spite of down-regulation of other inflammatory markers). Moreover, the compounds tested did not influence the phagocytosis stimulated with FITC-labelled S. aureus (Fig. 6B).

4. Discussion

Clinical and experimental data suggest that flavonoids and flavonoid-rich food exhibit a range of neuroprotective actions within the brain, including an ability to suppress neuro-inflammation, and the potential to promote memory, learning and cognitive functions (Vauzour et al., 2008). These substances have been also suggested to be capable to restore the functions of old microglia to their youthful state (Jang and Johnson, 2010). Moreover, the evidence obtained from both in vivo and in vitro models suggest that dietary flavonoids (including quercetin) are able to cross the blood brain barrier (BBB) and this process could be critically influenced by their physico-chemical properties, particularly, lipophilicity (Youdim et al., 2004a,b, 2003). In this regard, study of the BBB permeability of the brain-targeted drugs was influenced by their physico-chemical properties, particularly, lipophilicity (clogP 4.59 and clogP 2.16 for CPQ and Q, respectively (Milackova et al., 2015; Zizkova et al., 2014)). In addition, with regard to a possible enzymic or chemical hydrolysis of O-acyl moiety in vivo, CPQ may also serve as a prodrug with improved oral bioavailability enhancing the portion of quercetin penetrated into brain (Sun et al., 2009).

Neuroinflammatory processes in the CNS were shown to be associated with cell cycle protein expression in astrocytes, microglia (resulting in the increased proliferation) as well as neurons (preceding accelerated neuronal death) (Becker and Bonni, 2004). On the other hand, administration of cell cycle inhibitors, such as flavopiridol, roscovitine or olomoucine, were shown to provide neuroprotection in various in vitro as well as in vivo models (Byrnes et al., 2007). The cell cycle arrest of activated BV-2 microglia at G2/M phase was also shown for flavonoid apigenin (Eltsi et al., 2005). However, that was followed by a significant increase of apoptosis. Similarly to the finding, the phragmogelmin synthase inhibitor, tricyclodecan-9-yl-xanthogenate (D609), was shown to attenuate proliferation of resting BV-2 microglia without affecting viability, suggested to provide benefit in such conditions as a stroke (Gusain et al., 2012). Since microglia also play a role in many physiological processes in brain, preservation of their viability (along with suppression of their proliferation) might represent a desirable neuroprotective mechanism of CPQ.

Activated microglia and excessive production of NO by the inducible nitric oxide synthase (iNOS) were observed in various neurodegenerative diseases such as Parkinson’s and Alzheimer’s disease (AD) (Gonzalez-Scarano and Baltuch, 1999). Furthermore, pro-inflammatory cytokines were also found to play significant role in neurodegeneration (Hanisch, 2002). TNF-α and IFN-γ were shown to serve as stimuli for the appeal and activation of further microglial cells, thus promoting amplification of neurotoxic processes (Aschner et al., 1999; Hanisch, 2002). Therefore, novel pharmacological agents, which could inhibit iNOS expression and NO release as well as diminish cytokine production by microglia, represent a promising therapeutic approach to the control of potentially detrimental activity of microglia in neurodegeneration.

The more profound inhibitory effect of CPQ (on LPS-induced iNOS expression, NO and TNF-α release) is consistent with its predicted more efficient permeability through the cellular membranes and hence better biological availability. The anti-inflammatory effect of quercetin in endotoxin/cytokine-stimulated BV-2 microglia was shown to be accompanied by the down-regulation of extracellular signal-regulated kinase, c-Jun N-terminal kinase, p38, Akt, Src, Janus kinase-1, Tyk2, signal transducer and activator of transcription-1 (STAT-1), and nuclear factor-κB (NF-κB) (Kao et al., 2010). In addition, quercetin showed inhibitory effects on serine/threonine and tyrosine phosphatase activities and disrupted the accumulation of lipid rafts, the critical step for inflammatory signalling (Kao et al., 2010). Quercetin was also suggested to decrease nitrosative stress in LPS-stimulated BV-2 cells by suppressing NF-κB activation and inducing Nrf2-mediated heme oxygenase-1 (HO-1) expression (Kang et al., 2013). Furthermore, the study by Chen et al. (2005) showed that LPS-induced activation of IkB kinase (IKK), NF-κB and activating protein-1 (AP-1), and IFN-γ-induced activation of NF-κB, STAT1 and interferon regulatory factor-1 (IRF-1) in microglia were also lessened by quercetin.

Furthermore, our data suggest that iNOS downregulation can be
a cause of suppression of NO release (correlating with iNOS protein levels) more likely than a direct NO scavenging by the compounds tested. In support of this, in line with NO augmenting effects reported for quercetin both in vivo and in vitro (Loke et al., 2008), we found, paradoxically, an upregulation of nitrite levels in the system of NO chemically generated from sodium nitroprusside in the presence of CPQ and quercetin (10–35 μmol/l; data not shown).

Although many transcription factors such as NF-κB, C/EBPβ, AP-1, STAT, IRF-1, etc. play a relevant role in proinflammatory signalling, activation of NF-κB seems essential for the transcription of most of the proinflammatory molecules in microglia including iNOS and proinflammatory cytokines (Khasnativas et al., 2012). Therefore, attenuation of the activation of NF-κB, confirmed for both CPQ and quercetin, was suggested to be almost mandatory for a drug which exhibit anti-inflammatory effects.

NADPH oxidase in microglia as well as endothelial cells, astrocytes, and neurons can contribute to the production of superoxide in the brain. Generally, production of superoxide by myeloid cells is mediated by the phagocytic NADPH oxidase (Nox2). This enzyme is universally expressed in the brain (Infanger et al., 2006), however, its higher level may be found in microglia. Nox2 can be activated by various stimuli such as ATP, fibrillar amyloid β, prion protein or cytokines (TNF-α, INFγ, IL-1β) (Kraft and Harry, 2011). Although phagocytosis can also induce activation of Nox2, microglia do not uniformly undergo a respiratory burst when they initiate phagocytosis (Kraft and Harry, 2011; Savill et al., 2003). The microglial respiratory burst in response to receptor ligation involves opening of cell surface ion channels (such as P2X7 and Kv1.3) causing an “acute phase” of activation. This involves rapid protein kinase C (PKC)-dependent NADPH oxidase activation and superoxide release which in turn contribute to NF-κB activation (Kraft and Harry, 2011).

Microglial Nox is significantly implicated in the neurotoxicity associated with neurodegenerative disease. Excessive ROS production derived from activation of the Nox can be detrimental to both the microglia themselves and surrounding neurons and glia (Park et al., 2008). Glutathione depletion and oxidative stress resulting from the Nox-dependent respiratory burst was suggested to induce excitotoxic glutamate release from microglia causing neuronal loss (Barger et al., 2007). Microglial Nox activity also plays a role in the transition of microglial polarization to a proinflammatory phenotype (Bordt and Polster, 2014). On the other hand, inhibition of Nox promoted microglial polarization toward M2 anti-inflammatory phenotype while reducing the release of proinflammatory mediators (Choi et al., 2012b).

Both direct scavenging effect and inhibition of the enzymes involved in Nox signalling can contribute to the observed suppression of superoxide release by CPQ. Previous structure–activity relationship analysis revealed that flavonoids with unsubstituted catechol arrangement at the B ring (such as quercetin) can be superior O2− scavengers (Schewe et al., 2008). However, mono-O-substitution of catechol-type polyphenols changes the biological activity from superoxide scavengers to exclusive NADPH oxidase inhibitors. On the other hand, O2− scavenging efficacy as well as enzyme-inhibitory effects of quercetin (such as superior PKC inhibitory effect determined in a cell-free system by its unsubstituted catechol arrangement (Ferriola et al., 1989)) can be hindered by its less lipophilicity in comparison with CPQ. Furthermore, since iNOS and Nox were suggested to have synergistic effect in respiratory burst of the LPS-primed murine RAW264.7 macrophages, (Zhao et al., 2010), prominent downregulatory effect of CPQ on iNOS can also contribute to the suppression of O2− production in the LPS-pre-stimulated microglia.

Macrophages and microglia of middle-aged and older normal subjects were shown to physiologically perform amyloid β clearance, however this function was defective in patients with Alzheimer’s disease (Zhang et al., 2006). Several plant constituents such as curcumin (Bisht et al., 2009), green–tea polyphenols (Monobe et al., 2010) and ginger constituents (Koh et al., 2009) were shown to promote non-inflammatory phagocytosis in resting macrophages. Interestingly, treatment of macrophages from AD patients with curcuminoids increased their uptake of amyloid β (Zhang et al., 2006). Similar conditions can be observed during phagocytosis of apoptotic cells which do not provoke inflammatory signals, in macrophages (Voll et al., 1997).

Microglia can be driven to adopt M1 and M2 phenotypes, while M1 drives inflammation and M2 drives antiinflammatory processes and tissue repair (Cherry et al., 2014). Phagocytosis has essential function in both M1 and M2 phenotypes (Canton, 2014). However, it differs depending on the polarization. In contrast to M1 polarized cells (with a slower and less acidic phagosome beneficial for downstream immune functions), a rapid, more acidic phagosome helps M2 cells in quick and efficient elimination of debris (Canton, 2014; Cherry et al., 2014).

Both CPQ and quercetin preserved phagocytotic function of microglia with blocking NFκB activation and nitric oxide and cytokine synthesis in a non-inflammatory state. In this regard, both compounds tested upregulated the M2 phenotype marker to the level of resting cells. Therefore, a promotion of switch in macrophage phenotype from proinflammatory M1 to anti-inflammatory M2 may not be a reason of preservation of phagocytotic function.

In conclusion, our results point to the therapeutic potential of novel flavonoid derivative, 3′−O−(3-chloropivaloyl)quercetin (CPQ), in neurodegenerative diseases, mediated by favourable modulation of pro-inflammatory functions of microglia. Although CPQ did not show tremendous enhanced efficacy to mitigate inflammatory profile of BV-2 microglia compared to quercetin, its profound inhibitory effect on cell cycle progression together with enhanced lipophilicity (probably facilitating its availability in the brain), can rationalize suggested superior therapeutic prospective of this semisynthetic analogue of quercetin.

Conflicts of interest

The authors disclose that there is no conflict of interest.

Acknowledgement

The study was supported by VEGA 2/0031/12 and VEGA 1/0076/13. The work was supported by The Agency of the Ministry of Education of the Slovak Republic for the Structural Funds of EU, OP R&D of ERDF as a part of the Project: „Evaluation of natural substances and their selection for prevention and treatment of lifestyle diseases“ (ITMS 26240220040).

References


Bordt, E.A., Polster, B.M., 2014. NADPH oxidase- and mitochondria-derived reactive oxygen species in proinflammatory microglial activation: a bipartisan affair?

N. Mrvová et al. / Neurochemistry International 90 (2015) 246–254