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Dietary compounds prevent oxidative damage and nitric oxide production by cells involved in demyelinating disease

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Abstract

Oligodendrocytes and activated macrophages are involved in the immunopathology of demyelinating disease. In this study, we investigated the *in vitro* effect of dietary compounds, in particular flavonoids, on oxidative damage in OLN-93 oligodendrocytes and on nitric oxide (NO) production by NR8383 macrophages. Using a cell viability assay, we found the flavonoids luteolin and quercetin to protect OLN-93 cells against hydrogen peroxide-induced oxidative damage. Furthermore, apigenin and luteolin, but not morin inhibited NO production and reduced the expression of inducible NO synthase (iNOS) protein in lipopolysaccharide (LPS)-stimulated NR8383 macrophages. It was found that those dietary compounds effective in preventing oxidative damage in OLN-93 oligodendrocytes were not necessarily effective in reducing NO production and iNOS protein expression in NR8383 macrophages and vice versa. The different properties of the dietary compounds tested in this paper make them potential anti-inflammatory agents targeting neurodegenerative and neuroinflammatory diseases.

Keywords: Oligodendrocytes; Macrophages; Nitric oxide; iNOS; Oxidative damage; Polyphenols

1. Introduction

Various cell types are involved in the immunopathology of multiple sclerosis. Oligodendrocytes, the myelin producers of the central nervous system are in particular vulnerable to oxidative stress, cytokine toxicity and phagocytosis by macrophages [1]. Activated macrophages and microglia are capable of myelin phagocytosis, but produce also high levels of NO and H_2O_2 [2,3] that may be cytotoxic to other cells. H_2O_2 itself is not directly harmful, but its conversion to the reactive hydroxyl radical, makes H_2O_2 a potentially cytotoxic agent [4,5]. It has been established that high levels of H_2O_2 ($\geq$50 μM) can be cytotoxic for various cell types [4,5]. Oligodendrocytes are notably vulnerable to H_2O_2-induced oxidative damage. They have the highest rate of oxidative metabolic activity of any cell type in the brain [6]. In addition, oligodendrocytes have a particularly high intracellular iron concentration [6], rather weak antioxidant defence mechanisms [7], and extended lipid rich myelin sheets. These features make oligodendrocytes highly susceptible targets for oxidative stress or even cell death [7].

Fisher *et al.* [8] have reported that oxidative stress is associated with demyelination. In this study it was observed that macrophages from patients with multiple sclerosis have increased production of superoxide radicals and H_2O_2. Moreover, from EAE experiments, the animal model for multiple sclerosis, it has been shown that ROS, in particular H_2O_2, play a role in EAE pathogenesis [2,9].

Besides H_2O_2, activated macrophages and microglia can be a source of NO [3]. *In vivo*, NO can have cytotoxic properties during the inflammatory response when it is

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Abbreviations: NO, nitric oxide; iNOS, inducible NO synthase; LPS, lipopolysaccharide; H_2O_2, hydrogen peroxide; EAE, experimental allergic encephalomyelitis; WST-1, 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate; OH, hydroxyl; ROS, reactive oxygen species.

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produced at micromolar levels. In the cerebrospinal fluid of multiple sclerosis patients the level of NO metabolites, nitrite and nitrate, is significantly increased compared to controls [10,11]. Excessive nitric oxide production in the brain during inflammation may be detrimental to neurones and oligodendrocytes [12,13]. *In vivo*, aminoguanidine, an inhibitor of iNOS, has shown to ameliorate EAE in SJL mice [14]. On the other hand, administration of SIN-1, a nitric oxide donor, during the incipient phase also ameliorated EAE in Lewis rats [15]. Thus, the precise role of NO in EAE is still a matter of debate [16].

The current treatment of inflammatory demyelinating disease is aimed at immunosuppresive and immunomodulatory mechanisms. However, these therapeutic approaches are not suitable for all patients, because of irresponsiveness or undesired adverse reactions. Nutritional intervention might be an alternative and easy pathway to influence the course of disease beneficially. Little is known about the effect of dietary compounds on mechanisms described for inflammatory demyelinating disease. Besides the influence of dietary fat on multiple sclerosis [17], current research also aims at antioxidant intake and oxidative stress as important target areas [18,19]. We, therefore, set out to evaluate the effects of dietary compounds as an opportunity for a supportive treatment in inflammatory demyelinating disease.

Flavonoids and other dietary compounds like curcumin and apocynin are natural occurring plant metabolites that are part of our daily diet [20–22]. Dietary sources of flavonoids, which have been recognized as beneficial compounds against cardiovascular disease and associated with a reduced risk for other chronic diseases [20,21] are fruit, vegetables, grains, tea and wine [20]. Curcumin is isolated from turmeric root, and apocynin is extracted from the rhizomes of *Picrorhiza kurroa* [23]. Various bioactive properties have been described for these compounds. Besides antioxidant activity and direct free radical scavenging, anti-inflammatory, antiallergic, antiviral, and anticarcinogenic features have been reported [24–26].

In vitro effects of dietary compounds have been investigated in several cell lines [27–29]. Studies about the effect of dietary compounds on iNOS enzyme activity are contradictory [29,30] and effects on iNOS protein expression have been tested for only a limited number of dietary compounds [29,31]. In addition, the effect of dietary compounds on H_2O_2-induced damage in oligodendrocytes and the structure activity relationship derived from that has not been established before.

The aim of this study was to investigate the *in vitro* effect of dietary compounds on mechanisms of oxidative damage in oligodendrocytes and NO production and iNOS protein expression by activated macrophages. By testing dietary compounds with comparable molecular structures in two different *in vitro* models for oxidative damage and NO production, respectively, a structure activity relation could be determined.

2. Materials and methods

2.1. Reagents

Extracts with a high content of apigenin, hesperetin, luteolin, and quercetin were purchased from Kaden Biochemicals, apocynin was supplied by Sigma-Aldrich Chemie, curcumin by Aldrich Chemie Co. Morin, a synthetic compound was purchased from Indofine Chemical Company Inc. Class, molecular structure, and purity of the dietary compounds tested in this study are depicted in Table 1. Before use, dietary compounds were freshly dissolved in 50 mM KOH to a stock solution of 5 mg/mL. In the experiments, the stock solutions were further diluted in culture medium to maximal 1% (v/v) of the solvent. From pilot experiments, it was observed that the KOH vehicle did not affect cell viability or NO production.

Rabbit anti-mouse iNOS (Cayman Chemical Company), mouse anti-β-actin (Sigma-Aldrich Chemie), goat anti-mouse IgG-HRP and anti-rabbit IgG-HRP (both from Santa Cruz Biotechnology) were used for western blot experiments. All cell culture reagents were purchased from Invitrogen Life Technologies.

2.2. Cell culture

OLN-93 cells, rat oligodendrocytes [32] (kindly provided by Dr. H. de Vries, Department of Membrane Cell Biology, University of Groningen, The Netherlands), were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose and supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/mL penicillin, and 100 μg/mL streptomycin. A rat alveolar macrophage cell line, NR8383 (ATCC), was used to determine the effects of dietary compounds on NO production by macrophages. Cells were maintained in culture at a concentration of 5×10^5/mL of floating and adhering cells in RPMI 1640 medium containing 10% heat-inactivated FCS, 100 U/mL penicillin and 100 μg/mL streptomycin. All cells were grown in a humidified incubator at 37° with 5% CO_2.

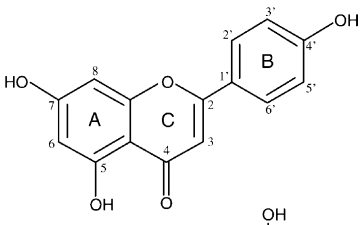
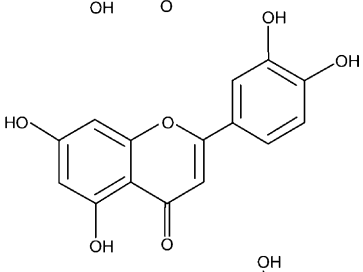
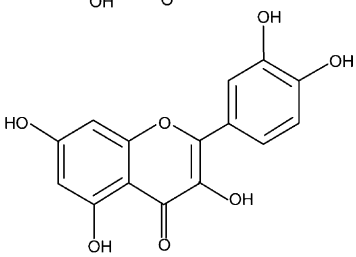
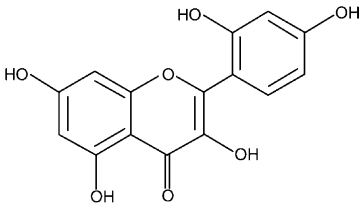
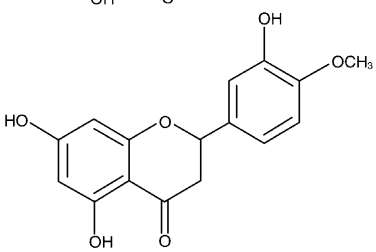
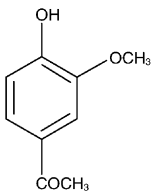
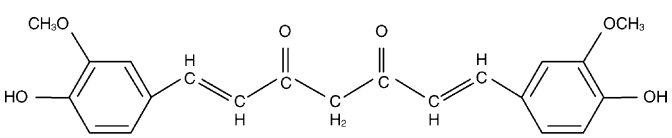
For protein isolation, cells were washed three times with prewarmed PBS. Total protein was harvested with Laemmli lysis buffer containing 2% SDS, 25% glycerol, and 63 mM Tris–HCl, pH 6.8. All samples were stored at −20° until analysis.

2.3. H_2O_2-induced oxidative damage in OLN-93

The effect of H_2O_2-induced oxidative damage on OLN-93 oligodendrocytes was measured using a WST cell viability assay. This colorimetric assay is based on the cleavage of the tetrazolium salt WST-1 (Boehringer) to formazan by mitochondrial dehydrogenases in viable cells.

OLN-93 cells were plated at 50,000 cells/well in 96 well microtiter plates. After overnight recovery, cells were incubated with 10^{-5} to 10^{-2} M H_2O_2 (Merck)

Table 1
Class, molecular structure, and purity of dietary compounds

Dietary compound	Class	Structure	Purity
Apigenin	Flavone		97.3% HPLC
Luteolin	Flavone		97.5% HPLC
Quercetin	Flavonol		99.7% HPLC
Morin	Flavonol		100% (synthetic)
Hesperetin	Flavanone		99.62% HPLC
Apocynin	Acetophenones		98% HPLC
Curcumin	Diferuloylmethane		65–70% HPLC

in the presence or absence of dietary compounds (2.5–25 μg/mL). Catalase (Sigma-Aldrich Chemie), an H_2O_2 detoxifying enzyme, was used as a control. Meanwhile WST-1 (1:10 final dilution) was added to each well and cell viability was measured spectrophotometrically at 450 nm (655 nm reference) after 3 hr incubation at 37° and 5% CO_2. Wells without cells were used to correct for background signal. Dose–response curves were plotted and analysed with GraphPad Prism (GraphPad Software Inc.).

2.4. LPS-induced NO production from NR8383 cells

For LPS-induced NO production experiments, NR8383 cells were plated in 96-well plates (5×10^4 cells/well) in a final volume of 100 μL culture medium. NO production was induced by adding 1 μg/mL of bacterial LPS (phenol extracted lipopolysaccharide from *Escherichia coli* 0111:B4; Sigma). During the 48-hr LPS stimulation, cells were incubated in the absence or presence of dietary compounds in final concentrations varying between 2.5 and 50 μg/mL. Aminoguanidine (Cayman Chemical Company), a well known inhibitor of iNOS enzyme activity, was used at a final concentration of 10^{-2} M as a positive control. After 48-hr incubation, 96-well plates were centrifuged to spin down nonadhered cells. The amount of nitric oxide produced by LPS-stimulated NR8383 cells was determined using the Griess-assay. In short, 100 μL of the cell supernatant was incubated for 5 min with 100 μL of Griess reagent, which contained 1% (w/v) sulphanilamide and 0.1% (w/v) *N*-(1-naphtyl)ethylenediamine dihydrochloride in 10.2% phosphoric acid. Standards containing 0–100 μM $NaNO_2$ in RPMI 1640 medium were used for quantification. Optical densities were measured at 550 nm using a microplate reader (BioRad). The toxicity of the tested compounds was determined in parallel experiments without LPS stimulation by the WST cell viability assay described above.

2.5. Western blot

Total protein was determined with the BioRad DC protein assay. Samples were equally loaded, normalized for protein content (5 μg per lane). A mouse macrophage-derived iNOS electrophoresis standard was applied according to the manufacturer's protocol (Cayman Chemical Company). Proteins were resolved by SDS-PAGE on 10% gels. Resolved proteins were transferred to PVDF membranes (Roche Diagnostics) in transfer buffer (25 mM Tris, 192 mM glycine, and 20% (v/v) methanol) containing 0.02% SDS. Membranes were blocked overnight at 4° in 1 M Tris, pH 8, 150 mM NaCl, and 0.05% Tween 20 (TBS-T) containing 5% Protifar Plus milk powder (Nutricia). Primary antibodies were incubated for 1 hr at room temperature (RT) in TBS-T. Polyclonal anti-iNOS and monoclonal mouse anti-β-actin antibody were both used at a dilution of 1:1000. Membranes were subsequently washed with TBS-T and incubated for 1 hr at RT with the anti-rabbit IgG- or goat anti-mouse IgG-HRP-labeled secondary antibodies at a dilution of 1:2000 or 1:7000, respectively. Immunoblots were washed with TBS-T, followed by two sequential wash steps using TBS without Tween 20 and then incubated with ECL chemiluminiscence reagents (Roche Diagnostics). Protein bands of iNOS and β-actin were visualised using the Lumi-Imager™ system (Boehringer). Changes in iNOS protein expression were analyzed by densitometry and expressed as arbitrary densitometry units. The protein expression of β-actin was used as an internal control.

2.6. Statistical methods

All values are expressed as mean ± standard error of the mean (SEM) of N observations. Treated groups were compared to untreated controls by using an unpaired *t*-test with unequal variances. Statistical analysis was done using SPSS statistical package (11.5.0; SPSS Inc.). In all cases, a P-value < 0.05 was considered significant.

3. Results

3.1. Effect of dietary compounds on H_2O_2-induced oxidative damage in OLN-93 oligodendrocytes

In pilot experiments, the optimal incubation time and the number of cells to evaluate the effect of H_2O_2 on the oligodendrocyte cell line OLN-93 was determined (data not shown). Cell viability was monitored by measuring the conversion of WST-1 to formazan during 1, 2, 3, and 4 hr at cell densities of 25,000, 50,000, 75,000, or 100,000 cells per well in the presence of a concentration range of 10^{-5} to 10^{-2} M H_2O_2. The optimal kinetics was reached after a 3-hr incubation of 50,000 cells per well with H_2O_2. A proper dose–response curve was obtained with a top plateau at 10^{-5} M H_2O_2 where no effect on the cell viability was observed and at 10^{-2} M H_2O_2 the bottom plateau was reached where the cell viability was severely reduced.

The range of doses (2.5–25 or 2.5–50 μg/mL) used for the dietary compounds tested in this paper was based on preliminary *in vitro* experiments with comparable compounds. From these experiments it was obvious that a dose higher than 50 μg/mL often showed detrimental effects on cell viability. At present however, little is known on the extrapolation of the *in vitro* doses to the *in vivo* systemic availability of these compounds after oral administration.

H_2O_2 exposure resulted in a dose-dependent reduction of oligodendrocyte cell viability (Fig. 1). Catalase, used as positive control, prevented H_2O_2-induced oxidative damage in the OLN-93 cells up to a concentration of 10^{-3} M H_2O_2. At a concentration of 10^{-2} M H_2O_2, the damage to the oligodendrocytes could no longer be prevented by catalase co-incubation. Of the tested dietary compounds, both luteolin and quercetin (see Fig. 1A and B, respectively) were able to prevent H_2O_2-induced oxidative damage significantly, while apigenin, apocynin, curcumin, and hesperetin had no effect in the tested dose range (data not shown). Statistic evaluation of the $-\log EC_{50}$ values revealed that luteolin effectively reduced the H_2O_2-induced oxidative damage in OLN-93 cells in a dose-dependent way from 2.5 to 10 μg/mL ($P < 0.01$) (Fig. 1A; Table 2). However, at a dose of higher than 25 μg/mL luteolin becomes cytotoxic to the oligodendrocytes, as established

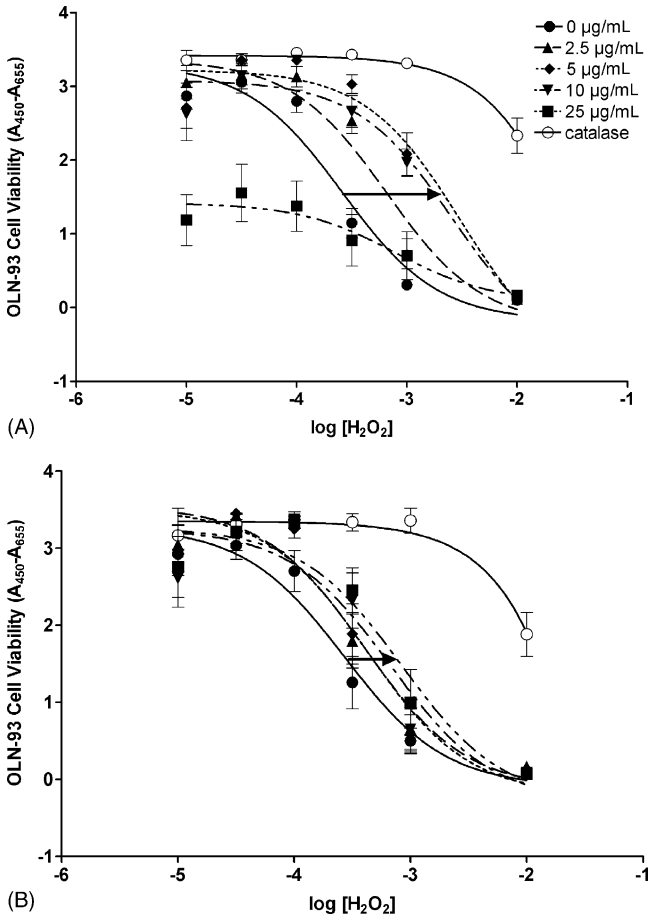


Fig. 1. Log dose–response curves of OLN-93 cell viability after 3 hr of H_2O_2-induced oxidative damage in the presence of various concentrations (2.5–25 μg/mL) of luteolin (A, N = 7) or quercetin (B, N = 6). Control curves were obtained from cells incubated without dietary compound in the absence or presence of 4000 U/mL catalase. The data are presented as sigmoidal dose–response curves of mean ± SEM. The arrows indicate the maximal rightward shift of the control curve at the $-\log EC_{50}$ point in the presence of 5 μg/mL luteolin or 25 μg/mL quercetin, respectively, revealing increased protection against oxidative damage.

by WST assay. Quercetin was a less potent protector of oxidative cell damage (Fig. 1B; Table 2) with $-\log EC_{50}$ values at concentrations of 10 and 25 μg/mL being significantly different from control value ($P < 0.05$). Interestingly, quercetin showed no deleterious effect on the OLN-93 cell viability in the same tested dose range.

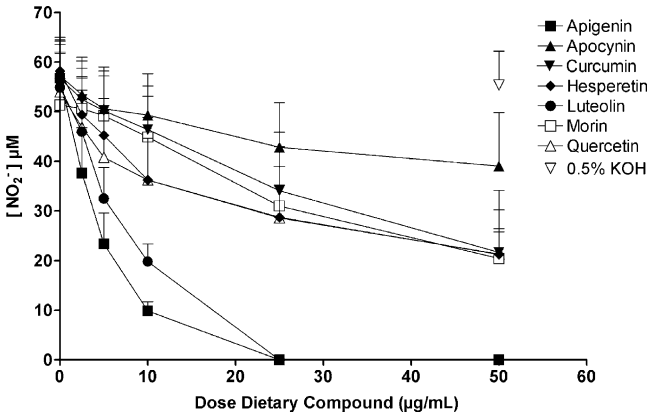


Fig. 2. Dose-dependent reduction of the nitrite production in 48-hr LPS-stimulated NR8383 cells incubated with various dietary compounds. Mean ± SEM of controls (0 μg/mL), dietary compounds (2.5–50 μg/mL), and the 0.5% KOH vehicle of four independent experiments are shown.

3.2. Effect of dietary compounds on LPS-induced nitrite production in NR8383

The effect of dietary compounds on LPS-induced NO production by NR8383 rat macrophages was tested. Stimulation of NR8383 cells for 48 hr with 1 μg/mL LPS resulted in a basal production of ~55 μM of nitrite, the stable end product of nitric oxide, while in the absence of LPS no nitrite could be detected (Fig. 2). Moreover, the dietary compound solvent, tested at the highest concentration of 0.5% 50 mM KOH, had no effect on the LPS-induced macrophage nitrite production. In the presence of LPS and 10^{-2} M aminoguanidine, a relative selective inhibitor of iNOS enzyme activity, nitrite production was adequately inhibited and remained below the detection limit (data not shown). Co-incubation with the various dietary compounds in a range of 2.5–50 μg/mL showed a dose-dependent reduction of the NR8383 produced nitrite concentration (Fig. 2). Apigenin and luteolin were the most potent dietary compounds that reduced, respectively, 80 and 60% of the nitrite production at a dose of 10 μg/mL. At 25 and 50 μg/mL, these dietary compounds completely blocked the nitrite production. However, when the dietary compounds were tested in parallel for their effect on NR8383 cell viability with a WST assay, it was shown

Table 2
Mean $-\log EC_{50}$ values ± SEM of H_2O_2 dose–response curves with OLN-93 cells in the presence or absence of dietary compound

	Mean $-\log EC_{50}$ ± SEM at different dosages of dietary compound				
Dietary compound	Control	2.5 μg/mL	5 μg/mL	10 μg/mL	25 μg/mL
Apigenin	3.59 ± 0.11	3.40 ± 0.13	3.41 ± 0.12	3.45 ± 0.14	3.62 ± 0.37
Apocynin	3.56 ± 0.09	3.41 ± 0.09	3.41 ± 0.10	3.44 ± 0.08	3.44 ± 0.10
Curcumin	3.52 ± 0.10	3.35 ± 0.10	3.34 ± 0.09	3.39 ± 0.13	3.50 ± 0.30
Hesperetin	3.62 ± 0.11	3.45 ± 0.12	3.37 ± 0.12	3.41 ± 0.13	3.48 ± 0.14
Luteolin	3.59 ± 0.11	3.18 ± 0.11*	2.51 ± 0.19*	2.59 ± 0.20*	3.10 ± 0.51
Quercetin	3.57 ± 0.15	3.40 ± 0.14	3.37 ± 0.15	3.17 ± 0.18**	3.08 ± 0.19**

* $P < 0.01$ for $-\log EC_{50}$ values at 2.5, 5, and 10 μg/mL of luteolin treated cells.
** $P < 0.05$ for $-\log EC_{50}$ values at 10 and 25 μg/mL of quercetin treated cells vs. control (0 μg/mL).

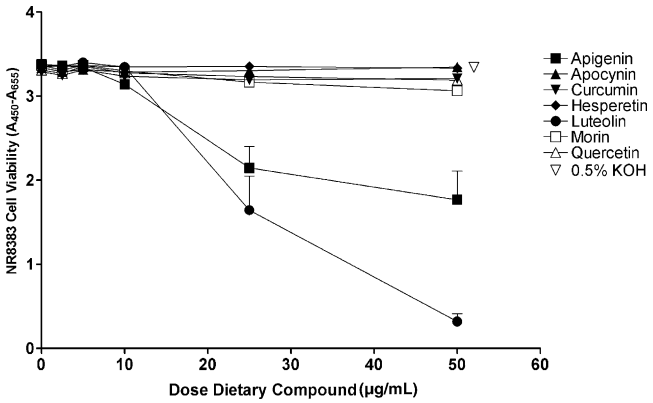


Fig. 3. Cell viability of NR8383 macrophages incubated for 48 hr with various concentrations of dietary compound as determined by WST. Both luteolin and apigenin compromise cell viability at higher concentrations (≥25 μg/mL). Mean ± SEM of four independent experiments are shown.

that at these high concentrations both apigenin and luteolin have detrimental effects on macrophage viability (Fig. 3). The other dietary compounds nor their KOH vehicle were cytotoxic to the NR8383 cells at any concentration tested. Hesperetin, morin and quercetin showed a less pronounced, but significant reduction of LPS-induced nitrite production ([hesperetin] ≥ 25 μg/mL: $P < 0.05$; [morine] ≥ 50 μg/mL: $P < 0.01$; [quercetin] ≥ 50 μg/mL: $P < 0.05$ vs. control). In addition, apocynin and curcumin reduced the nitrite production by NR8383 in a dose-dependent fashion, however this did not reach significance.

3.3. Effect of dietary compounds on iNOS protein expression

The effect of the flavonoids apigenin, luteolin and morin on the expression of iNOS was examined to further unravel the mechanism by which these compounds reduce NO production. From pilot experiments we learned that iNOS protein expression in LPS-stimulated NR8383 increased during a time span of 8–24 hr and decreased after 48 hr. The iNOS protein expression of the 24-hr-stimulated macrophages in the presence or absence of dietary compounds was slightly higher compared to the 8-hr data with a similar expression pattern. Therefore, we show the results of the 8-hr experiments (Fig. 4). Apigenin and luteolin are

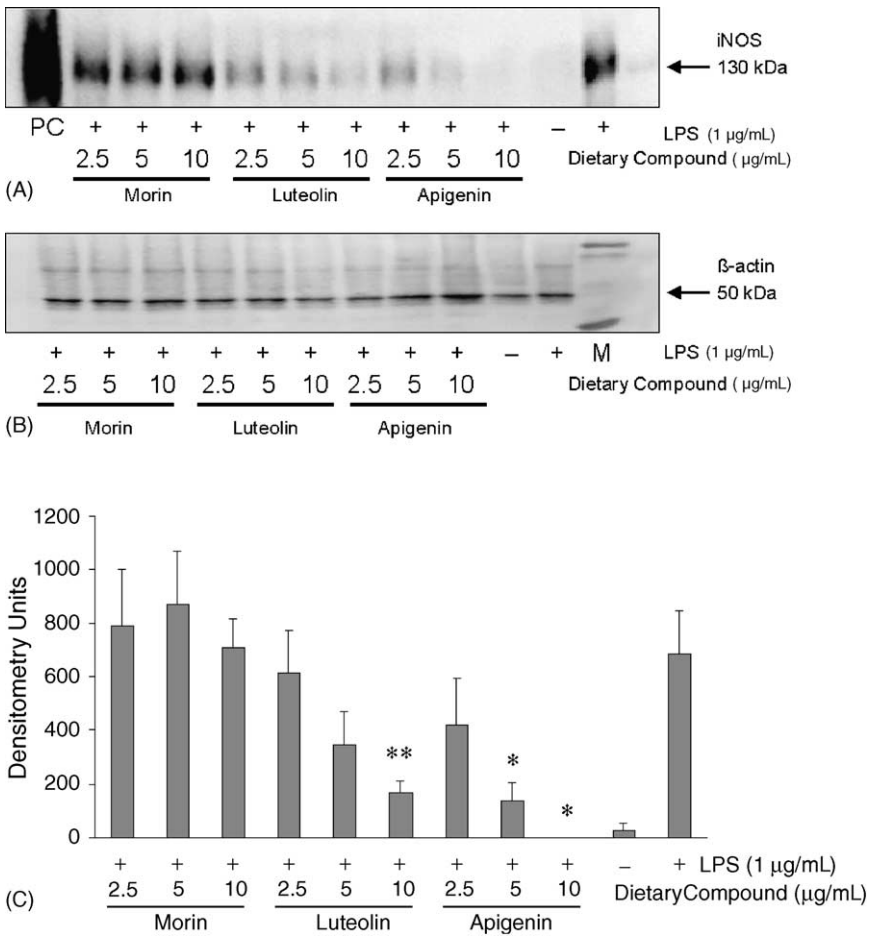


Fig. 4. Representative western blot of (A) iNOS protein expression and (B) β-actin housekeeping protein expression in NR8383 cells stimulated with LPS during 8 hr in the presence or absence of apigenin, luteolin or morin. PC: positive control; M: marker. Apigenin and luteolin dose-dependently reduced iNOS protein without affecting the β-actin housekeeping protein expression. (C) Densitometry units of four independent experiments (mean ± SEM; $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ vs. LPS-stimulated control).

both very strong reducers of LPS-induced macrophage NO production, while morin was used as an example of a less potent reducer (see also Fig. 3).

After 8-hr LPS-stimulation iNOS protein expression was increased in NR8383 cells. Aminoguanidine, the relative selective inhibitor of the activity of the iNOS enzyme obviously had no effect on the iNOS protein expression. Of the tested dietary compounds, both apigenin and luteolin reduced the iNOS protein expression significantly in a dose-dependent fashion. In accordance with its lagged capacity to prevent LPS-induced macrophage NO production, morin had no significant effect on the iNOS protein expression in the tested concentrations. The protein expression of β-actin was used as an internal control and was not affected by the different experimental treatments.

4. Discussion

H_2O_2, a ROS produced by activated macrophages and microglia cells in the central nervous system has deleterious effects on myelin-forming oligodendrocytes. In rat EAE, the animal model for multiple sclerosis it has been demonstrated that *in vivo* treatment with catalase, an H_2O_2 scavenger, suppressed the severity of disease [2]. Also optic neuritis could be suppressed in murine EAE by adeno-associated viral mediated catalase expression [9]. Reduction of H_2O_2-induced cell damage may be an interesting target mechanism in neuroinflammatory disease.

Here we investigated the *in vitro* effect of dietary compounds on H_2O_2-induced oxidative damage in oligodendrocytes. H_2O_2-induced oxidative damage to OLN-93 cells was established as decreased cell viability during a 3-hr exposure in the presence or absence of dietary compounds. The results demonstrated that luteolin and quercetin could effectively protect against this H_2O_2-induced oxidative damage in a dose-dependent manner. However, apigenin, apocynin, curcumin and hesperetin did not prevent H_2O_2-induced oxidative damage in OLN-93 cells in the tested concentration-range.

Flavonoids, apocynin, and curcumin all have in common a (poly)phenolic molecular structure (see Table 1). Furthermore, hesperetin, apocynin, and curcumin are also structured with one or two methoxy groups and additionally curcumin has a 1,3-diketone system. Due to these different structures, dietary compounds will exert different modes of action and effects. Of the flavonoid compounds, it is known that the hydroxylation positions at the A and B ring are important for their antioxidant activity [26]. Luteolin and quercetin both have 4-OH groups equally distributed at the A and B ring. Moreover, quercetin has an extra 3-OH group at the C ring, but apparently this is not a suitable property to reduce H_2O_2-originating free radical cell damage. When the molecular structures of luteolin and apigenin are compared, the only difference between luteolin and apigenin is the presence of a 3′-OH group at the B ring in luteolin. This 3′,4′ dihydroxy substitution of luteolin at the B ring is essential to prevent H_2O_2-derived free radical-induced cell damage, since, in contrast to luteolin, apigenin does not show this effect. Hendriks *et al.* [33] recently published supporting findings regarding flavonoid structure and bioactivity in an *in vitro* model of macrophage myelin phagocytosis. ROS, like H_2O_2, are known to be involved in myelin phagocytosis processes. Similar to our findings both luteolin and quercetin were effective in this model. These compounds showed strong inhibition of myelin phagocytosis and significantly reduced cellular ROS production, while apigenin and hesperetin were less effective.

NO is another agent that can induce cell damage. The mode of action for cellular damage by NO involves decreased anti-oxidant defence of glial cells [34], inhibition of mitochondrial function, DNA single-stranded breaks [35], and formation of the very reactive peroxynitrite, a strong oxidizing and nitrating agent in the presence of superoxide [7,12,36]. In this study, we tested the capacity of dietary compounds to reduce NO production after macrophage activation. The results show a strong reduction of LPS-activated NR8383 macrophage-derived NO by apigenin and luteolin at low doses. At high apigenin and luteolin concentrations impaired cell viability occurred. The dietary compounds apocynin and curcumin did not show significant effects on NR8383 macrophage NO production, while hesperetin, morin, and quercetin were only effective at higher dosage without affecting cell viability.

A reduced NO production may be the result of direct NO scavenging, inhibition of iNOS enzyme activity, inhibition of iNOS protein synthesis or blockade. Effects of dietary compounds on these different levels have been investigated in several other cell lines. Kim *et al.* [29] have found inhibitory effects of flavonoids on NO production from LPS-activated murine RAW 264.7 macrophages. With decreasing potency amongst others, luteolin > apigenin > quercetin > morin were effective when tested in a dose range of 10–100 μM (~3–34 μg/mL). In addition, flavonoids have also been shown, besides NO production, to repress TNF-α secretion and NF-κB-dependent gene expression in IFN-γ-stimulated RAW 267.4 macrophages [28]. In 24-hr LPS/IFN-γ stimulated rat C6 astrocytes, NO production was reduced with decreasing potency by quercetin > morin > curcumin > apigenin > hesperetin [27]. The different observations in our study on flavonoid and curcumin effectiveness may be due to differences between cell lines, the method of macrophage stimulation as well as the source and purity of the compounds.

In subsequent experiments, the effect of dietary compounds on 8-hr LPS-induced iNOS protein expression in the NR8383 cells was studied. Addition of apigenin and luteolin, the most potent inhibitors of NO production in our study, resulted in a decreased iNOS protein expression. Morin, a less potent inhibitor of NO production, did not

show significant effects on the expression of the iNOS enzyme in the tested concentrations. Thus, it can be concluded that the flavonoids apigenin and luteolin act on macrophage-derived NO production by reducing iNOS protein expression. However, from these experiments it can not be excluded that direct NO scavenging or inhibition of iNOS enzyme activity also partly contributes to the dietary compound reduced macrophage NO production.

Apocynin, the nonflavonoid dietary compound is extensively used as an anti-inflammatory agent in Asian medicine [23]. Furthermore, it has been described to inhibit the production of ROS by NADPH oxidase in activated human neutrophils and macrophages [37,38] and *in vivo* it has been shown that oral administration of apocynin is effective in an experimental animal model for human collagen arthritis [24]. Because of its structure, we expected at least some radical scavenging capacity of apocynin. However, in our hands apocynin was not effective in the *in vitro* models used in this study. Apparently the anti-inflammatory action of apocynin is mediated via NADPH oxidase inhibition, a different mechanism that was not studied in our experimental approach.

Curcumin, frequently used as a yellow pigment in curry, has been demonstrated to have anti-inflammatory and anticarcinogenic activity [39]. Moreover, curcumin has various antioxidant properties due to its particular structure [40]. The present results show that curcumin was not effective in preventing H_2O_2-induced oligodendrocyte cell damage and it reduced the LPS-stimulated macrophage-derived NO production in the tested dose range, but not significantly. The latter results are in line with the results of Chan *et al.* [40]. They showed that curcumin inhibited LPS- and IFN-γ-induced NO production by mouse peritoneal cells at 2.5–10 μM.

The applicability of dietary compounds like flavonoids, curcumin and apocynin in human chronic inflammatory disorders deserves further study with special reference to the systemic bioavailability of these compounds since little is known about the absorption and biotransformation of such compounds. Nevertheless, there is growing evidence from human feeding studies that the absorption and bioavailability of specific flavonoids is much higher than originally believed [41]. Several studies have shown that quercetin glycosides are clearly absorbed in healthy subjects provided with black tea or onions as a flavonoid source [42] or after direct supplementation with the isolated forms [43]. Positive effects of dietary compounds have also been observed in several experimental animal models. The flavonoids luteolin and quercetin[1] and curcumin [44] strongly inhibited clinical signs in EAE. However, these effects were observed after i.p. administration, hence bypassing the gastrointestinal tract. In a study by Pan *et al.* [45], a single oral administration of curcumin in BALB/c mice resulted in poor absorption from the gut as well as rapid plasma clearance, traces of curcumin could still be observed in the brain. In humans, the ability of dietary compounds to cross the blood brain barrier is largely unknown. Therefore, extensive studies to determine the distribution of dietary compounds when absorbed from the gut are needed. In conclusion, it was found that dietary compounds are effective in preventing H_2O_2-induced oxidative damage in OLN-93 oligodendrocytes and in reducing NO production by activated NR8383 macrophages *in vitro*. Reduction of NO production by LPS-activated macrophages by luteolin and apigenin was associated with a decreased expression of iNOS protein. The dietary compounds tested in this paper have versatile properties in modulating oxidative damage and NO production. This makes them interesting candidates as anti-inflammatory agents in specific clinical nutrition targeting neurodegenerative and neuroinflammatory diseases.

[1] Hendriks, personal communication.

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