Chapter 2

The combination of vitamins and omega-3 fatty acids has an enhanced anti-inflammatory effect on microglia

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Abstract

Neuroinflammation is a common phenomenon in the pathology of many brain diseases. In this paper, we explore whether selected vitamins and fatty acids known to modulate inflammation exert an effect on microglia, the key cell type involved in neuroinflammation. Previously these nutrients have been shown to exert anti-inflammatory properties acting on specific inflammatory pathways. We hypothesized that combining nutrients acting on converging anti-inflammatory pathways may lead to enhanced anti-inflammatory properties as compared to the action of a single nutrient. In this study, we investigated the anti-inflammatory effect of combinations of nutrients based on the ability to inhibit the LPS-induced release of nitric oxide and interleukin-6 from BV-2 cells. Results show that omega-3 fatty acids, vitamins A and D can individually reduce the LPS-induced secretion of the pro-inflammatory cytokines by BV-2 cells. Moreover, we show that vitamins A, D and omega-3 fatty acids (docosahexaenoic and eicosapentaenoic) at concentrations where they individually had little effect, significantly reduced the secretion of the inflammatory mediator, nitric oxide, when they were combined. The conclusion of this study is that combining different nutrients acting on convergent anti-inflammatory pathways may result in an increased anti-inflammatory efficacy.

1. Introduction

Neuroinflammation is a natural response of the innate immune system of the brain to disturbances of homeostasis, such as pathogen invasion and tissue damage. When neuroinflammation becomes chronic and excessive, it can have detrimental effects on brain function. Neuroinflammation is a phenomenon that is observed during progression of several psychiatric and neurodegenerative disorders, such as depression, schizophrenia, Alzheimer’s disease and Parkinson’s disease [1–6].

Epidemiological studies point towards a beneficial effect of specific diets on the incidence of brain diseases in which neuroinflammation plays a role [7–9]. In recent years, there has been increasing interest in investigating nutrients that could be responsible for these protective effects [10–12].
Since neuroinflammation in a common phenomenon in various brain disorders, it is compelling to investigate nutrients in our diet which exert anti-inflammatory properties. *In vitro* studies serve as a first step in the selection of substances to be considered for further investigation *in vivo*. *In vitro* studies allow preliminary screening of diverse nutrients to assess their potential anti-inflammatory action when acting directly on particular cell types. However, the anti-inflammatory properties of a single nutrient at relevant concentrations are usually small and insufficient to achieve an adequate therapeutic effect. Combining different nutrients acting on convergent inflammatory pathways may therefore be helpful in avoiding the necessity of using high concentrations of one component. Elucidation of mechanisms of action of potentially anti-inflammatory nutrients can facilitate the design of effective combinations of those nutrients.

Microglia cells are thought to be the most important cell type involved in neuroinflammation. The aim of this study was therefore to evaluate the incremental anti-inflammatory effects of combinations of selected nutrients on microglia cells. In particular, we aimed to investigate whether a combination of nutrients which are known to act via diverse anti-inflammatory pathways could be more effective in inhibiting the pro-inflammatory effect of LPS on microglia cells than each individual component in the mixture. We evaluated the anti-inflammatory effect of the nutrients in BV-2 cells, which is an immortalized cell line derived from C57bl/6 mice and is thought to closely resemble the physiology of primary microglia cells *in vitro* [13]. Lipopolysaccharide (LPS), an endotoxin derived from the cell wall of gram-negative bacteria, is the most widely used agent for activation of immune cells.

This study consists of three parts. First, the anti-inflammatory effect of single nutrients was investigated. Second, the mechanisms of action of those individual nutrients that showed efficacy, but for which the mechanism was not reported in the literature yet, was studied. In the final part of the study, the additive effects of combined nutrients, which act on convergent anti-inflammatory pathways, was evaluated.
2. Material and methods

2.1 Reagents

The following substances were purchased from Sigma Aldrich and used without further purification: lipopolysaccharide (LPS, from Escherichia coli 055:B5, cat # L6529), vitamin B_{6} (cat # P5669-5G), B_{9} (folic acid, cat # F7878), vitamin B_{12} (cat # V6629), vitamin A (cat # 95144), all-trans retinoic acid (RA, cat # R2625), vitamin D_{3} in a form of 7-dehydrocholesterol/vitamin 25(OH)D_{3} (100µg/ml in ethanol, cat # 47763), cis-4,7,10,13,16,19-docosahexaenoic acid (DHA, cat # D2534), cis-5,8,11,14,17-eicosapentaenoic acid (EPA, cat # E7006), valproic acid (VPA, cat # P4543), S-(5′-adenosyl)-L-homocysteine (SAH, cat # A9384), anacardic acid (ANA, cat # A7236). Inhibitors of retinoic acid receptors (RAR) were purchased from Tocris Bioscience: BMS 195614 (4-[[[5,6-dihydro-5,5-dimethyl-8-(3-quinolinyl)-2-naphthalenyl]carbonyl]amino]benzoic acid, cat # 3660), LE 135 (4-(7,8,9,10-tetrahydro-5,7,10,10-pentamethyl-5H-benzo[e]naphtho[2,3-b][1,4]diazepin-13-yl)benzoic acid, cat # 2021), MM 11253 (6-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1,3-dithiolan-2-yl]-2-naphthalene-carboxylic acid, cat # 3822). The concentrations of nutrient stock solutions used in the experiments are listed in Table 1.

2.2 BV-2 cell culture

BV-2 cells were obtained from IRCCS (Azienda Ospedaliera Universitaria San Martino – IST Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy). Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM, Gibco) supplemented with 10% of heat-inactivated fetal bovine serum (Invitrogen), 10,000 units/ml penicillin (Gibco), 10,000 µg/ml streptomycin (Gibco) and 200 mM L-glutamine (Gibco) and maintained at 37°C and 5% CO_{2}. Twenty-four hours before the experiment, cells were plated on 96 well plates (Corning) at a cell density of approximately 20,000 cells/well (i.e. approximately 50% confluency) and maintained in DMEM supplemented with 2% fetal bovine serum, 10,000 units/ml penicillin 10,000 µg/ml streptomycin and 200 mM L-glutamine for the duration of the experiment.
2.3 Cell viability

Cell proliferation and viability were assessed with 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT, Sigma) at a concentration of 1 mg/ml. Phenazine methosulfate (PMS, Sigma) at a concentration of 200 mg/ml was added as an electron coupling reagent. The XTT/PMS solution in sterile water (100 µl) was added to each well. After 2h, the absorbance was measured at 450 nm (and 690 nm as a reference). The absorbance of untreated controls was considered as 100% viability, whereas viability for each condition (x) was calculated as follows: \[
\frac{\text{absorbance}(x)}{\text{absorbance}(\text{untreated controls})} \times 100.
\]
The viability test was repeated for each experiment as a within plate control. The results of experiments, in which the cell viability was below 85% of that of the untreated control cells, were excluded from the data analysis.

2.4 Griess assay (nitric oxide release)

Cells were treated with either a single nutrient or a combinations of nutrients for 24 h. This treatment was followed by exchanging the medium for fresh medium with LPS (50 ng/ml) combined with the same nutrients. In addition, two control conditions were included in each experiment: i) cells that were neither treated with nutrients nor LPS (viability control) and ii) cells that were only treated with LPS, but not with nutrients (LPS control). After incubation for 24 h, media were collected for measurement of nitric oxide (NO) release. The stable product of NO released by the BV-2 cells is NO\(_2^\cdot\). The concentration of NO\(_2^\cdot\) was measured with a Griess assay Kit (Promega) according to the manufacturer’s instructions. Data were corrected for cell viability (the viability of untreated control cells represented 100% of viability). Data are represented as percentage of the NO released by cells stimulated only with LPS (LPS control). To assess variability between the tests, data from LPS controls were pooled and the average NO release between experiments was calculated. For LPS controls, the NO release is presented as the percentage of the average release between experiments.

2.5 ELISA (IL-6, IL-4 and IL-10)

Cells were treated with either a single nutrient or a combinations of nutrients for 24 h. Subsequently, the medium was removed and cells were incubated for 24 h in fresh medium with LPS (50 ng/ml) combined with the same nutrients. In addition, two control conditions were included in each experiment: i) cells that were neither
treated with nutrients nor LPS (viability control) and \( ii \) cells that were only treated with LPS, but not with nutrients (LPS control). Media were collected for the measurement of IL-6, IL-4 and IL-10. Collected samples were stored at -80 °C until further analysis. The cytokine levels were assessed using ELISA MAX™ Deluxe from Biolegend according to the manufacturer’s protocol. Data were corrected for cell viability (the viability of untreated control cells represented 100% of viability). Data are presented as percentage of the IL-6 released by cells stimulated only with LPS. To assess variability between the tests, data from LPS controls were pooled and the average IL-6/IL-4/IL10 release between experiments was calculated. For LPS controls, the IL-6 release is represented as the percentage of the average release between experiments.

2.6 Mechanistic studies

In order to investigate the mechanism of action of vitamin A, BV-2 cells were treated with various inhibitors (VPA (500 µM), ANA (1 µM), SAH (100 µM), BMS 195614 (6 µM), LE 135 (1 µM), MM 11253 (2 µM)) 3 h prior to the addition of 1.75 µM vitamin A or 10 µM retinoic acid, RA. This treatment was followed by 24 h incubation with LPS (50 ng/ml) combined with vitamin A or RA and one of the inhibitors. Media were collected for measurement of NO and IL-6 release. Data were corrected for cell viability (the viability of untreated control cells represented 100% of viability). Data are represented as percentage of the NO/IL-6 released by cells stimulated only with LPS. To assess variability between the tests, data from LPS controls were pooled and the average NO/IL-6 release between experiments was calculated. For LPS controls, the NO/IL-6 release is presented as the percentage of the average release between experiments.

2.7 Statistical analysis

All experiments were performed at least in triplicate. Data are presented as dots representing the mean and bars representing the standard error of the mean (SEM). Due to the small sample size, non-parametric statistical tests were used. Data obtained from testing single nutrients were analyzed with the Kruskal-Wallis H test and if statistical significance was reached, a Mann Whitney U test with Bonferroni-Holm post-hoc test was performed to correct for multiple comparisons.
Data from the evaluation of RAR inhibitors (figure 2) were analyzed by paired comparisons between conditions with and without the inhibitor. Therefore, in this case the analysis was performed only with the Mann Whitney U test.

An effect sizes (r) was included in the analysis of data in fig 2 and in supplementary fig 2. The effect size for each comparison was calculated according to the following equation: $r = \frac{Z}{\sqrt{N}}$, where N is the total number of repetitions and Z is the z-value obtained from the Mann Whitney U test.

A difference was considered statistically significant when the probability (p) was <0.05. An effect size was considered as small when $r \leq 0.1$, medium when $0.1 \leq r \leq 0.3$, large when $0.3 \leq r \leq 0.5$ and very large when $r > 1.3$. [14].

### 3. Results

3.1 Effect of individual nutrients on the production of pro-inflammatory cytokines by LPS-activated BV-2 cells.

In the first part of the study, we tested the effects of single nutrients on the LPS-induced activation of BV-2 cells. The nutrients were used in high, but non-toxic concentrations: i.e. concentrations at which cell viability does not decrease below 90% (dose vs. viability curves not shown). The release of NO by activated BV-2 cells was measured with the Griess assay. Cells exposed to the culture medium or to the medium supplemented with only nutrients did not release any detectable amounts of NO (data not shown). On the other hand, exposure of BV-2 cells to LPS (50 ng/ml) for 24 h induced a strong release of NO. Vitamins B$_6$, B$_9$ and B$_{12}$, and the amino acids L-tryptophan and L-cysteine did not cause any significant effect on LPS-induced NO release in these experiments (data not shown). As shown on the figure 1A, the fatty acid EPA caused decreased the LPS-induced NO release to 80% of the NO released by LPS controls (IQR, 75-91%, p=0.115), but this effect was not statistically significant yet. In contrast, DHA and the combination of DHA and EPA did significantly decrease LPS-induced NO release to 68% (IQR, 49-77%, p=0.036) and 61 % (IQR 43-66 %, data not shown) relative to LPS controls, respectively. Both vitamins A and D (fig 1 A) significantly decreased the NO release to 43% (IQR 36-55%; p=0.032) and to 39% (IQR 20-52.4%; p=0.024) of the NO release by LPS controls, respectively.
In a similar manner, the anti-inflammatory effect of the nutrients on LPS-induced IL-6 release by BV-2 cells was investigated with ELISA. LPS treatment caused a concentration-dependent increase in IL-6 release from BV-2 cells (data not shown). Vitamins B₆, B₉ and B₁₂, and the amino acids L-tryptophan and L-cysteine did not cause any significant effects on LPS-induced IL-6 release in these experiments (data not shown). As depicted on fig 1B, the fatty acid EPA decreased in the LPS-induced IL-6 release by BV-2 cells to 80% the NO release by LPS controls (IQR, 67-96%, p=0.7, n=4), although this decrease did not reach statistical significance. DHA and the combination of DHA and EPA, on the other hand, did significantly decrease LPS-induced NO release to 68% (IQR 49-77%, p=0.032, n=4) and 51% (IQR 38-54%, data not shown), respectively. Also vitamins A and D significantly decreased IL-6 release to 42% (IQR 39-58%, p=0.024, n=4) and 43% (IQR 21-57%, p=0.024, n=4) of LPS controls, respectively (fig 1B).

Taken together, these data demonstrate that vitamins A and D – and to a lesser extent DHA and EPA – show an anti-inflammatory effect on LPS-stimulated BV-2 cells. The B vitamins and the amino acids L-cysteine and L-tryptophan did not have an anti-inflammatory effect on LPS-activated BV-2 cells.

3.2 Effect of individual nutrients on the production of anti-inflammatory cytokines by LPS-activated BV-2 cells

To investigate whether the moderating effect of individual nutrients on the secretion of pro-inflammatory cytokines was accompanied by a shift of the BV-2 cells to an anti-inflammatory phenotype, the release of the anti-inflammatory cytokines IL-4 and IL-10 was measured. However, none of the investigated nutrients, except EPA, was able to induce any significant increase in the secretion of these cytokines by LPS-stimulated BV-2 cells. The IL-4 release from cells treated with LPS alone or LPS combined with nutrients was below detection limit of the assay used (data not shown), while the IL-10 release was detected (supplementary figure 1). Only treatment with EPA increased significantly the release of IL-10 from LPS-activated BV-2 cells (130%, IQR 89-116%, p=0.012). The treatment with vitamin A caused a trend towards increased IL-10 release (127%, IQR 101-161%, p=0.014), however this effect did not reach statistical significance due to the large variations.
3.3 Effects of RAR inhibitors on the anti-inflammatory efficacy of vitamin A.

Literature data indicate that the anti-inflammatory activity of omega-3 fatty acids and vitamin D is mediated by several nuclear receptors, including the proliferator-activated receptor, retinoic X receptor, and vitamin D receptor [15–17]. However, no data are available about the mechanism of action of the anti-inflammatory effect of vitamin A. To investigate a potential epigenetic mechanism of action, we evaluated the effect of chemical inhibitors of histone acetylation, deacetylation or methylation on the anti-inflammatory action of vitamin A, but did not find any significant effect on NO or IL-6 release (supplementary data, fig 1). Therefore, we also tested whether pharmacological inhibition of retinoic acid receptors (RARα, β and γ) can reverse the anti-inflammatory effect of vitamin A.

As shown in fig. 2A and 2B, the RARα inhibitor BMS 195614 could reverse the anti-inflammatory action of vitamin A, resulting in a change in NO release by LPS-activated microglia from 46% (IQR, 42.2-59.8%) to 103% (IQR 91.5-117.6%) of the NO released by LPS-treated control cells (U<0.001, Z=-2.3, p=0.029, r=0.82, n=4). Similarly, BMS 195614 reversed the anti-inflammatory effect of RA (from 49% (IQR 39.2-58.6%) to 117% (IQR 98.1-135.3%), U<0.001, Z=-2.3, p=0.029, r=0.82, n=4). BMS 195614 also reversed the anti-inflammatory effect of vitamin A (from 63% (IQR 44.0-77.2%) to 94% (IQR 84.9-100.3%), U=1.000, Z=-2.02, p=0.057, r=0.71) and RA (from 82% (IQR 58.8-96.1%) to 130% (IQR 102.9-151.3%), U<0.001, Z=-1.96, p=0.1, r=0.8, n=4) on the release of IL-6 by LPS-activated BV-2 cells. Although these effects on IL-6 release were not statistically significant, large effect sizes were observed.

The RARβ inhibitor LE 135 could not reverse the anti-inflammatory effect of vitamin A and RA on NO or IL-6 release, but LE 135 by itself did significantly reduce the effect of LPS on the IL-6 release (73.9 % (IQR, 67.9-78.8), U=3.000, Z=-2.4, p=0.014, r=1.2, n=4).

The RARγ inhibitor MM 11253 also did not affect the anti-inflammatory effect of vitamin A RA on NO or IL-6 release. However, MM 11253 had an anti-inflammatory effect by itself, as it significantly blocked both the NO release (43% (IQR, 41.5-47.7%); U<0.001, Z=-2.9, p=0.001, r=1.45, n=4) and IL-6 release, (79.7 (IQR, 75.97-82.6%); U=3.000, Z=-2.0, p=0.049, r=1.0, n=4) induced by LPS alone.
Together these results suggest that the anti-inflammatory effect of vitamin A is mediated by a pathway downstream of RARα.

3.4 Effect of combinations of nutrients on LPS-induced activation of BV-2 cells.

The most effective nutrients investigated in this study were vitamins A and D and the fatty acids DHA and EPA. The combination of fatty acids with vitamins A and D decreased the NO release by BV2 cells to 10% (IQR, 0.6-20.4%) and the IL-6 production to 21% (IQR, 11-35%) as compared to the levels secreted by LPS-stimulated controls (data not shown). However, after post-hoc correction the effect of the combined nutrients was not significantly different from the effect obtained with single nutrients. The high nutrient concentrations used in this experiment probably already caused a strong anti-inflammatory effect for individual nutrients, which leaves a small window of opportunity for detecting a further decrease in cytokine release upon treatment with a combination of nutrients.

3.5 Effect of combinations of nutrients on LPS-induced activation of BV-2 cells after decreasing the nutrient concentrations.

We decreased the concentrations of vitamin A and D and the fatty acid DHA to a level in which they did not have a significant effect on the release of NO as compared to the LPS-stimulated controls (Table 1). The EPA concentration did not need to be changed. As shown in fig. 3, lower concentrations of single nutrients hardly caused any effect on NO or IL-6 release. The combination of these substances in the same concentrations, on the other hand, caused a significantly larger reduction in NO release (to 37% of the secretion by LPS-stimulated control cells (IQR 28.0-45.7%), p=0.032, Bonferroni-Holm post-hoc correction) than any of the individual nutrients. The combination nutrients also resulted in a larger decrease in IL-6 release (to 84% (IQR, 54.1-88.9) of the release by LPS-stimulated controls), but this effect was not statistically different (p>0.05) from the effects of single nutrients.

4. Discussion

In the first part of this study we tested the efficacy of single nutrients on the inhibition of LPS-induced release of pro-inflammatory markers (NO and IL-6) by
BV-2 cells. Some of the nutrients investigated, or their active metabolites, have already been shown to exert an anti-inflammatory effect in similar in vitro models of activated microglia [19–21]. However, here we have investigated not only the effect of individual components, but also combinations of them.

First, we tested the ability of a high concentration of a particular nutrient to attenuate NO release by LPS-stimulated BV-2 cells. In activated immune cells, NO is produced from L-arginine by a reaction catalyzed by iNOS. The expression of iNOS is mainly activated by the transcription factor NF-κB [22]. Excessive NO production has been shown to cause a neurotoxic effect [23]. NO mediates glutamate neurotoxicity in several brain pathologies, including neurodegenerative diseases [24]. The ability to suppress excessive NO release by activated immune cells is widely used as an indicator of anti-inflammatory efficacy. Our experiments showed that high concentrations of vitamins A and D and the fatty acid DHA are able to inhibit LPS-induced NO release by BV-2 microglia cells. Vitamins B₆, B₉ and B₁₂ and the amino acids L-tryptophan and L-cysteine did not cause any significant attenuation of NO production in these cells.

Similar results were obtained when IL-6 released by BV-2 cells was measured. We have chosen this marker of proinflammatory response, because previous studies have demonstrated elevated serum levels of IL-6 in animals after exposure to noxious stimuli and in patients with diverse inflammatory diseases. Increased levels of IL-6 were correlated with disease severity. Therefore, some of the new treatment strategies for inflammatory diseases are directed at inhibiting the IL-6 signaling pathway [25,26]. In the present study we tested the ability of nutrients to inhibit LPS-induced IL-6 release in activated microglia. Our results show that vitamins A and D and the fatty acid DHA significantly inhibit the LPS-induced IL-6 release by these cells. Amino acids and B vitamins did not cause a significant alteration in the IL-6 release. We explored also the possible effects of nutrients on the release of anti-inflammatory cytokines by LPS-induced BV-2 cells. In our experiments, we did not observe any effect of investigated nutrients on IL-4 release, due to the fact that this cytokine was not detected in any cells treated with LPS (alone or with combination with nutrients). We observed an increase of IL-10 by cells treated with EPA.

Based on the effect of single nutrients on the release of pro-inflammatory markers we concluded that:
(I) Of the nutrients tested, vitamins A and D are the most efficient in suppressing inflammatory response in LPS-activated BV-2 cells. These results are consistent with previous studies showing that vitamins A and D can suppress proinflammatory response in activated immune cells [19,27].

(II) The fatty acid DHA showed a significant anti-inflammatory effect as measured by NO and IL-6 release in LPS-stimulated BV-2 cells, a result that is consistent with previous studies using a similar model [28]. The fatty acid EPA did not cause a significant attenuation of proinflammatory marker release in this study. However, combining EPA with DHA enhanced efficacy of DHA.

(III) B vitamins (B$_6$, B$_9$ and B$_{12}$) and the amino acids L-cysteine and L-tryptophan did not show a significant effect on LPS-induced activation of BV-2 cells, despite these substances having previously been described as inducing anti-inflammatory effects in vivo [29–31]. Our results suggest that the anti-inflammatory effects of B vitamins and the amino acids L-cysteine and L-tryptophan are probably due to other mechanisms than direct interaction with activated microglia cells, because in vitro studies usually only investigate the effect of a nutrient on one cell type, while the brain is much more complicated and permits the action of substances on many different pathways and the interaction between cell types.

In the second part of the study, we focused on the possible mechanism of action responsible for the anti-inflammatory effects of the nutrients tested. Many studies have shown that nuclear receptors play an important role in the regulation of the inflammatory response by nutrients [10,32,33]. The omega-3 fatty acids DHA and EPA have been shown to act on several nuclear receptors, such as peroxisome proliferator-activated receptors (PPARs), retinoic X receptors (RXR), nuclear receptor NF-κB and nuclear factor (erythroid-derived 2)-like 2 (Nrf2). PPARs are involved in the regulation of inflammatory response [34]. For example, PPARγ blocks the LPS-induced replacement of co-repressors by co-activators of pro-inflammatory gene expression [15]. Moreover, a recent study showed the involvement of PPAR β and δ in the anti-inflammatory action of retinoic acid in carrageenan-induced paw oedema in rats [35]. RXR is a nuclear receptor which forms dimers with RAR and PPAR. DHA acts as endogenous agonist of RXR [36]. DHA was shown to be a ligand for PPARα in vascular smooth muscle cells [16]. NF-κB is a transcription factor which is very important for the activation of proinflammatory genes in the early phase of an inflammatory response. EPA was shown to inhibit the release of proinflammatory mediators (such as NO, IL-6,
TNFα) from LPS-stimulated BV-2 microglia via inhibition of NF-κB activation [21]. Moreover, a recent study showed that both DHA and EPA inhibit NF-κB in LPS-stimulated endothelial cells [37]. There is evidence that inhibition of NO by DHA and EPA is also mediated by heme oxygenase 1 (HO-1). Blocking of HO-1 reversed the inhibitory effect of DHA on interferon γ-induced NO production in BV-2 cells [28]. Moreover, the HO-1 inducer, hemin, showed anti-inflammatory properties by inhibiting NO production in BV-2 cells [38]. HO-1 expression depends on the Nrf2 transcription factor. The Nfr2/HO-1-dependent pathway is stimulated by several anti-inflammatory compounds, such as quercetin or linalool [39,40].

The active metabolites of vitamins A and D are ligands for the retinoic acid receptors (RAR) and the vitamin D receptors (VDR), respectively. Vitamin D is metabolized in microglial BV-2 cells to its active form, 1,25-dihydroxy-vitamin D3, by enzyme 1α-hydroxylase. 1,25-dihydroxy-vitamin D3 inhibits the NO release from LPS-stimulated BV-2 cells via interaction with VDR [17]. VDR functions as a nuclear hormone receptor responsible for the repression or activation of numerous genes, including the genes encoding proinflammatory mediators. For example, vitamin D was shown to be involved in the inhibition of NF-κB activity in murine macrophages (RAW264.7) [41].

It is known that retinoic acid, the active metabolite of vitamin A, is a ligand for RAR. We investigated which RAR subtype mediates the anti-inflammatory action of vitamin A in BV-2 cells using selective inhibitors of RARα, RARβ and RARγ. BMS 195614 has been described as a selective inhibitor of RARα in HeLa cells [42], LE 135 was described as an antagonist of RARβ in human scleral fibroblasts [43] and MM 11253 was proven to be an antagonist of RARγ in human squamous cell carcinoma cells [44]. The treatment of BV-2 cells with BMS 195614 significantly reversed the effect of vitamin A and RA on the NO release by LPS-stimulated microglia cells. BMS 195614 also had a large effect size on the LPS-induced IL-6 release by BV2 cells. Our results indicate that RARα plays a pivotal role in the anti-inflammatory action of vitamin A on LPS-activated BV-2 cells. These results are consistent with previous studies showing the involvement of RARα signaling in the suppression of proinflammatory cytokine release from activated human T cells [45].

Our experiments also showed the possible involvement of RARγ receptors in the LPS-induced proinflammatory cytokine production by BV-2 cells. The inhibitor of
RARγ signaling, MM 11253, did not change the anti-inflammatory effect of vitamin A or RA, but it did reduce the NO and IL-6 release from BV-2 cells after LPS stimulation by approximately 50%. This finding is consistent with previous studies showing that RARγ is involved in the activation of proinflammatory gene expression, and that blocking of these receptors in vitro caused attenuation of LPS-induced TNFα release by mouse macrophages. This effect, however, was not observed in vivo in RARγ deficient mice, suggesting that RARγ signaling deficiency in vivo can be compensated by other mechanisms [46]. Compensating mechanisms in vivo have also been observed for downregulation of the expression of RARα. Mice in which RARα expression was partly downregulated with an antisense RARα DNA construct showed a compensatory effect in RARβ and RARγ receptor expression. This effect, however, was not observed in knockout mice in which RARα expression is completely abrogated [47]. As the in vivo effect of the blocking of RARα signaling might be compensated by other mechanisms, it is important that the results of this study, which suggest an important role for RARα signaling in attenuating the inflammatory response by microglia, are confirmed in vivo in an appropriate animal model. This can be done by dietary intervention changing the vitamin A supply (single component or in combination with other nutrients), by applying selective RARα ligands, such as antagonist BMS 195614 or agonist AM580 [45], or by using RARα knockout mice.

Reversible epigenetic changes, including DNA methylation, histone acetylation and deacetylation, have been shown to play an important role in the modulation of inflammatory response. However, our study did not show any evidence for the involvement of DNA methylation (inhibited by SAH), histone acetylation (inhibited by ANA) or histone deacetylation (inhibited with VPA) in the anti-inflammatory action of vitamin A, suggesting that vitamin A probably does not act via an epigenetic mechanism.

Taken together, it can be concluded that the nutrients investigated in this study affect various signaling pathways, mainly via nuclear receptors which affect the expression of inflammatory mediators.

In the third part of the study, we combined the nutrients with the most potent anti-inflammatory activity (vitamins A and D and the fatty acids DHA and EPA) with the aim to increase the overall anti-inflammatory efficacy. When the combinations of nutrients were used in the same concentrations as when studying the individual components, a trend towards an increased anti-inflammatory effect of the
combination as compared to the single components was observed. Since high concentrations of vitamin A and D and DHA already had a strong anti-inflammatory effect when used as a single nutrient, there was only a small window of opportunity to detect any additive effect of combining the nutrients.

We used lower concentrations of vitamin A and D and DHA to increase the detection window for our assays. Concentrations causing no significant changes in release of NO (as compared to LPS-stimulated controls) were selected based on the dose-effect curves. At these low concentrations of nutrients, the mixture of vitamin A and D and fatty acids DHA and EPA caused a significantly larger decrease in NO release than any of the single components. This convergence between food components is a promising concept to be tested in future studies, because only low concentrations of nutrients have to be used. The components investigated in this study and their combination are candidates for the future studies on prophylactic or therapeutic effects of dietary intervention aimed at neuroinflammation.

In conclusion, the most important finding of the present study is the increased anti-inflammatory activity that can be achieved when vitamin A and D and the fatty acids DHA and EPA are combined. This suggests that these substances act on convergent pathways. As a result, a combination of these nutrients can increase the anti-inflammatory effect without increasing the concentration of the individual components. Such a combination of food components could be used to design a dietary intervention aimed at preventing brain diseases in which neuroinflammation is involved. Since neuroinflammation is usually already present before symptoms appear, preventive dietary intervention could be included as integral part of a healthy lifestyle. When symptoms are already present, dietary intervention may still be used, either by itself or as an addition to an already available treatment in order to augment its effect.

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**Figures and tables:**

**Table 1** The list of nutrients, solvents and concentration of stock solutions, which were prepared fresh for each experiment. Concentrations and combinations used for the experiment. 1 - concentration used for experiments on figure 1, 2 and 3, 2 - concentration used for experiments on figure 4.

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Abbreviation</th>
<th>Solvent</th>
<th>Stock concentration</th>
<th>Treatment concentration</th>
<th>Concentration already present in the culture medium</th>
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<td>Vitamin B₆</td>
<td>B₆</td>
<td>water</td>
<td>10 mMᵃ</td>
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<td>Folic acid</td>
<td>B₉</td>
<td>1M NH₄OH</td>
<td>15 mMᵇ</td>
<td>15 µM</td>
<td>9 µM</td>
</tr>
<tr>
<td>Vitamin B₁₂</td>
<td>B₁₂</td>
<td>water</td>
<td>0.2 mMᵃ</td>
<td>0.2 µM</td>
<td>-</td>
</tr>
<tr>
<td>Docosahexaenoic acid</td>
<td>DHA</td>
<td>ethanol:F BS (1:4)</td>
<td>20 mMᵇ</td>
<td>20 µMᶜ</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6.67 µMᵈ</td>
</tr>
<tr>
<td>Eicosapentaenoic acid</td>
<td>EPA</td>
<td>ethanol:F BS (1:4)</td>
<td>20 mMᵇ</td>
<td>20 µMᶜᵈ</td>
<td>-</td>
</tr>
<tr>
<td>Vitamin A</td>
<td>A</td>
<td>ethanol</td>
<td>875 mMᵇ</td>
<td>1.75 µMᶜ</td>
<td>-</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td>0.583 µMᵈ</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>D</td>
<td>ethanol</td>
<td>10 mg/mlᵇ</td>
<td>1 µg/mlᶜ</td>
<td>-</td>
</tr>
<tr>
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<td></td>
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<td></td>
<td></td>
<td>0.1 µg/mlᵈ</td>
</tr>
</tbody>
</table>

ᵃ Solution kept at 4°C until incubation
ᵇ Solution kept at -20°C until incubation
ᶜ Concentrations used for experiments in figure 1, 2
ᵈ Reduced concentrations used for experiments in figure 3.
**Figure 1** The effect of single nutrients on LPS-induced NO and IL-6 release. BV-2 cells were pretreated for 24 h with different nutrients. This was followed by 24 h incubation with LPS (50 ng/ml) combined with the same nutrients. Media were collected for measurement of NO release with the Griess assay (A) and IL-6 release with ELISA (B). NO and IL-6 levels are presented as the percentage of the concentration produced by cells treated with LPS alone (LPS control). The level of NO/IL-6 release from control treated only with LPS (100%) is indicated on each graph with the dashed line. All graphs represent the results from 4 independent experiments (mean ±SEM). Data were statistically analyzed with Kruskal-Wallis H test, followed by a Bonferroni-Holm post-hoc test. A difference was considered statistically significant when p < 0.05 (p values from that test are mentioned on upper right part of each graph) and indicated as significantly different as compared to the LPS control group (*).

**Figure 2** The effect of BMS 195614, LE 135 and MM 11253 on the efficacy of vitamin A inhibition of LPS-induced NO and IL-6. BV-2 cells were pretreated for 3 h with one of the following inhibitors: BMS 195614 (6 µM); LE 135 (1 µM); MM 11253 (2 µM). This was followed by 24 h incubation with one of the inhibitors and vitamin A (1.75 µM) or RA (10 µM) followed by 24 h incubation of
LPS (50 ng/ml) combined with vitamin A or RA and the same inhibitor. Media was collected for measurement of NO (Griess assay) and IL-6 release (ELISA). Data is presented as the percentage of inflammatory mediator concentration released by cells treated with LPS. The level of NO/IL-6 release from controls treated only with LPS (100%) is indicated on each graph with the dashed line. All graphs represent the results from at least three independent experiments (mean ±SEM). Data were statistically analyzed with Mann Whitney U test and effect sizes were calculated. A difference was considered statistically significant when p < 0.05 and indicated with an asterisk (*).
**Figure 3** The effect of a combination of nutrients on LPS-induced NO and IL-6 release after decreasing the nutrient concentrations. BV-2 cells were pretreated for 24 h with single nutrients or their combination. This was followed by 24 h incubation with LPS (50 ng/ml) combined with the same nutrients. Media were collected for measurement of NO release with the Griess assay (A) and IL-6 release with ELISA (B). NO and IL-6 levels are presented as the percentage of the concentration produced by cells treated only with LPS (LPS control). The level of NO and IL-6 for LPS control (100%) is indicated on each graph with the dashed line. All graphs represent the results from five independent experiments (mean ±SEM). Data were statistically analyzed with Kruskal-Wallis H test. A difference was considered statistically significant when p<0.05 (p values from that test are mentioned on upper right part of each graph). To test for differences between the single nutrients and the mixture containing vitamin A, D and fatty acids DHA and EPA, a post hoc test with Bonferroni-Holm correction was performed. A difference was considered statistically significant when p < 0.05 and is indicated with an asterisk (*).
Supplementary data

**Figure 1** The effect of single nutrients on LPS-induced IL-10 release. BV-2 cells were pretreated for 24 h with different nutrients. This was followed by 24 h incubation with LPS (50 ng/ml) combined with the same nutrients. Media were collected for measurement of IL-10 release with ELISA (B). IL-10 levels are presented as the percentage of the concentration produced by cells treated with LPS alone (LPS control). The level of IL-10 release from control treated only with LPS (100%) is indicated on each graph with the dashed line. All graphs represent the results from 4 independent experiments (mean ±SEM). Data were statistically analyzed with Kruskal-Wallis H test, followed by a Bonferroni-Holm post-hoc test. A difference was considered statistically significant when p < 0.05 (p values from that test are mentioned on upper right part of each graph) and indicated as significantly different as compared to the LPS control group (*).

![Graph showing the effect of single nutrients on LPS-induced IL-10 release.](image)

**Figure 2** The effect of VPA, ANA and SAH on the efficacy of vitamin A inhibition of LPS-induced NO and IL-6. BV-2 cells were pretreated for 3 h with one of the following inhibitors: VPA (500 µM), ANA (1 µM), SAH (100 µM). This was followed by 24 h incubation with one of the inhibitors and vitamin A (1.75 µM) followed by 24 h incubation of LPS (50 ng/ml) combined with vitamin A and the
same inhibitor. Media were collected for measurement of NO release with the Griess assay (A, C, E) and IL-6 release with ELISA (B, D, F). Data is presented as the percentage of inflammatory mediator concentration released by cells treated with LPS. The level of NO/IL-6 release from control treated only with LPS (100%) is indicated on each graph with the dashed line. All graphs represent the results from at least three independent experiments (mean ±SEM). Data were statistically analyzed with Mann Whitney U test and effect sizes were calculated. A difference was considered statistically significant when p < 0.05 and indicated with an asterisk (*).
Chapter 2

References:


Enhanced anti-inflammatory effect of vitamins and omega-3 fatty acids


