The impact of nutrition on neuroinflammation in vitro and in vivo
Kurtys, Ewelina Anna

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2017

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Chapter 4

The anti-inflammatory properties of rice bran components on LPS-stimulated microglia

Ewelina Kurtys¹, Ulrich L. M. Eisel², Tom Metz¹, Robert J. J. Hageman³, Martin J. Verkuyl³, Laus M. Broersen³, Rudi A. J. O. Dierckx¹, Erik F. J. de Vries¹

¹ Department of Nuclear Medicine and Molecular Imaging, University of Groningen, University Medical Center Groningen, PO Box 30.001, 9700 RB Groningen, The Netherlands.
² Department of Molecular Neurobiology, Center for Life Sciences, University of Groningen, Groningen, The Netherlands
³ Nutricia Research, Uppsalalaan 12, 3584 CT Utrecht, The Netherlands
Abstract

Rice bran contains a wide range of substances, which can exert beneficial properties for health. Therefore, there is increasing interest in further investigation of these substances and in a potential use of them, for example as modulators of inflammation. In this study, we explored different combinations of rice bran components known to exert anti-inflammatory properties. Furthermore, we combined rice bran components with selected vitamins and fatty acids, known to modulate inflammation. The goal of the study was to test whether combining diverse nutrients leads to enhanced anti-inflammatory properties, as compared to single nutrients. The anti-inflammatory effect was evaluated based on the ability of the compounds to inhibit the lipopolysaccharide-induced release of NO and IL-6 from BV-2 cells. Results show that the investigated nutrients were able to inhibit LPS-induced NO release, especially geranylgeranyl pyrophosphate and ferulic acid. Moreover, combining rice components with vitamin D, at concentrations where they individually had little effect, resulted in a trend towards enhanced anti-inflammatory efficacy. Thus, combining different nutrients acting on the convergent anti-inflammatory pathways may lead to an increased anti-inflammatory effect.
1. Introduction

Rice is the major component of the diet for a large part of the human population and provides more than 20% of the calories consumed by humans worldwide. Rice bran, the outer layer of a whole brown rice kernel, is often considered as a by-product of rice processing and is discarded as waste or used as animal food. However, rice bran contains high amounts of various nutrients, including proteins, fats, dietary fibres, minerals, anti-oxidants and anti-inflammatory phytochemicals [1]. Bioactive phytochemicals from rice bran have been reported to exert various beneficial properties [2]. Animals studies have shown that rice bran extracts can have a modulatory effect on inflammatory processes and thus can diminish the progression of symptoms in disease models for obesity [3], colitis [4], LPS-induced systemic inflammation [5], and D-galactose-induced ageing [6,7]. Recent findings indicate that rice bran extracts can also have beneficial effects on brain function. For example, supplementation of the diet with rice bran components had a positive impact on biochemical and neuropsychological parameters in patients with minimal hepatic encephalopathy [8,9].

Gamma oryzanol (γ-OZ) is the most intensively studied phytochemical from rice bran. γ-OZ is a lipid-soluble secondary plant metabolite with strong antioxidant and anti-inflammatory properties [3,10–12]. For example, recent studies in a rat model of high-fat and high-fructose diet-induced metabolic syndrome has demonstrated that γ-OZ supplementation effectively decreased serum proinflammatory markers [13]. Ferulic acid (FA) is the carboxylic acid part of the phytosterol ester γ-OZ. FA is present not only in rice bran, but also in other plants such as grain, citrus fruit and some vegetables [14]. Recent findings have shown a therapeutic effects of FA in the middle cerebral artery occlusion model in rats, in which it enhanced neuroprotection and decreased proinflammatory markers in the brain following ischemia [15–17]. Evidence also suggest a role of FA in modulation of brain function. FA was shown to have an anti-depressant effect in mice through modulation of the serotonergic system [18]. Geranylgeraniol (GGOH) and one of its metabolites, geranylgeranyl pyrophosphate (GGPP), are isoprenoids that are present in rice bran and other plants. GGOH and GGPP can play an important role in modulation of the inflammatory processes. For instance, in animals intraperitoneally injected with LPS, dietary supplementation with GGOH reduced plasma inflammatory markers [19].
Microglia cells are considered key players of neuroinflammation as they produce and release a wide range of mediators of inflammatory processes, such as cytokines, chemokines and nitric oxide (NO). The release of proinflammatory mediators is used as a measure of the inflammatory response of microglia cells to the noxious stimuli both in vitro and in vivo. The ability to inhibit microglia activation is considered a characteristic of the anti-inflammatory properties of therapeutic substances. In this study, we investigated whether combinations of bioactive phytochemicals from rice bran can have a synergistic anti-inflammatory effect on activated microglia in vitro. Here, we used the BV-2 microglia cell line, an immortalized cell line derived from C57Bl/6 mice, thought to closely resemble the physiology of primary microglia cells and therefore a well-established model for investigation of anti-inflammatory activity of potential therapeutic drugs in vitro [20]. BV-2 cells were stimulated with lipopolysaccharide (LPS), an endotoxin derived from the cell wall of gram-negative bacteria, to provoke the release of pro-inflammatory mediators. The anti-inflammatory potency of \( \gamma \)-OZ, FA, GGOH, GGPP and combinations thereof on LPS-stimulated BV-2 cells was evaluated by measuring the release of the inflammatory mediators nitric oxide (NO) and interleukin-6 (IL-6). In addition, rice bran components were combined with vitamins A and D and the fatty acids cis-4,7,10,13,16,19-docosahexaenoic acid (DHA) and cis-5,8,11,14,17-eicosapentaenoic acid (EPA). These fatty acids and vitamins were previously shown to exhibit anti-inflammatory properties ([21–24]). Here, we wanted to investigate whether additive or synergistic anti-inflammatory effects between these nutrients and rice bran components could be achieved.

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), \( \gamma \)-OZ, GGPP, trans-FA, GGOH, vitamin A, vitamin D\(_3\) in a form of 7-dehydrocholesterol/vitamin 25(OH)D\(_3\) (100\( \mu \)g/ml in ethanol), DHA and EPA. The initial combinations and concentrations of \( \gamma \)-OZ, GGPP, FA and GGOH (as MIX A, B and C) are presented in Table 1.
2.2 BV-2 cell culture

BV-2 cells were purchased 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) at 37°C in an atmosphere with 5% CO₂. Twenty-four hours prior the experiment, cells were plated in 96 well plates (Corning) at a cell density of approximately 20,000 cells/well and maintained in the same culture medium, with a lower concentration (2%) of heat-inactivated fetal bovine serum.

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 cells for each condition was corrected for the absorbance of untreated control cells (considered as 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 for 24 h with different combinations of nutrients. This treatment was followed by replacing the medium with fresh medium with LPS (50 ng/ml) combined with the same nutrients. After incubation for another 24 h, media were collected for measurement of NO release. The concentration of nitrite (NO₂⁻), which is the stable product of NO released by the BV-2 cells, 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 presented as percentage of the NO released by cells stimulated with only LPS. To assess variability between the tests, data from the treatment with LPS alone were pooled and an average release between experiments.
was calculated for NO. For LPS alone treatment the NO release is represented as the percentage of the average release between experiments.

2.5 ELISA (IL-6)

Cells were treated for 24 h with different combinations of nutrients. Subsequently, the medium was removed and cells were incubated for another 24 h in fresh medium with LPS (50 ng/ml) combined with the same nutrients. Media were collected for measurement of IL-6 release. 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 represented as percentage of the IL-6 released by cells stimulated with only LPS. To assess variability between the tests, data from the treatment with LPS alone were pooled and an average release between experiments was calculated for IL-6. For LPS alone treatment the IL-6 release is represented as the percentage of the average release between experiments.

2.7 Statistical analysis

Statistical analysis was performed using IBM SPSS software Statistics 22 (SPSS Inc., United States). All experiments were performed at least four times. Data are presented as horizontal lines representing means and bars representing standard error of the mean (SEM). Due to the small sample size, non-parametric statistical tests were used. Data were analyzed with the Kruskal-Wallis H test and if the statistical significance was reached, a Mann Whitney U test with a Bonferroni-Holm post-hoc correction for multiple comparisons was performed. A difference between groups was considered statistically significant when the probability (p) value was <0.05.

3. Results

3.1 The effect of combinations of γ-OZ, GGPP, FA and GGOH on LPS-induced NO and IL-6 release from BV-2 cells.

First, viability tests were performed in order to determine the highest nontoxic concentration for each individual compound: i.e. the concentration at which cell
viability is at least 90% as compared to untreated controls (data not shown). Next, substances were combined into three mixes (MIX A, B and C), in which the concentration of one component was near the highest non-toxic concentration and the other compounds were added in the same proportion as present in rice bran (Table 1). Then, a dose-effect curve of NO release due to LPS treatment was obtained, in order to select the half maximal effective concentration (EC50). Stimulation of BV-2 cells with LPS (50 ng/ml) for 24 h caused a robust release of NO, as measured with the Griess assay. Cells exposed only to the culture medium or to the medium supplemented with the nutrients only did not release any detectable amounts of NO (data not shown). In order to investigate the potency of nutrients to inhibit the LPS-induced NO release, 24 h pre-treatment with nutrients followed by co-treatment of the same nutrients with LPS for another 24 h was applied on BV-2 cells.

The MIX A combination with all rice components investigated in this study (γ-OZ, GGPP, FA and GGOH) did not cause any significant decrease in the LPS-induced NO release, but instead caused an increase in NO release from LPS-stimulated BV-2 cells when administered at a 100-fold dilution (100-fold diluted MIX A: 119%, IQR, 111.0-125.0, U=2.000, Z=-2.378, p=0.048) (fig 1A). γ-OZ is present in rice bran in much higher amounts than the other investigated nutrients. As a consequence, the concentrations of GGPP, FA and GGOH in MIX A were >2000-fold lower than the highest nontoxic concentrations of these nutrients (Table 1).

The next combination, MIX B, contained the highest nontoxic concentration of GGPP and two other components, FA and GGOH, in the same proportion as they occur in rice. MIX B caused a significant decrease of NO release to 62% as compared to LPS-stimulated controls (IQR, 62-65%, U<0.001, Z=-2.932, p=0.006). The anti-inflammatory properties of MIX B were so potent that even a 100-fold dilution could significantly decrease the LPS-induced NO release to 90.5% (IQR, 87-91%, U<0.001, Z=-2.722, p=0.008) (fig 1B). However, when comparing the potency to inhibit NO release by MIX B and one of its single components, GGPP (used in the same concentration as in MIX B), no significant difference was observed (MIX B vs. GGPP U=5.000, Z=-0.750, p=0.571). GGPP alone decreased NO release to 83.7% (IQR, 65.8-88.2%, GGPP vs. LPS: U=0.000, Z=-2.449, p=0.012) as compared to the LPS-treated controls (fig 1D).
The next combination, MIX C, contained the highest nontoxic concentration of FA and one other component, GGOH, in the same proportion as they occur in rice bran. This combination significantly decreased NO release to 35.5% (IQR, 28.0-49.5%, U<0.001, Z=-2.722, p=0.012). Further dilutions of MIX C did not have any significant effect on NO release (fig 1C). However, similarly to the MIX B, the anti-inflammatory effect of MIX C was not significantly different from the anti-inflammatory effect of one of its components, FA (fig 1D). FA alone decreased NO release to 36.2% (IQR, 26.9-41.8%, U<0.001, Z=-2.449, p=0.012) as compared to LPS-stimulated controls. This effect was not significantly different from the effect of MIX C (FA vs. MIX C: U=5.000, Z=-0.357, p=0.857).

Next, the effect of the investigated nutrient combinations on LPS-induced IL-6 production by BV-2 cells was investigated (fig 1 E-H). LPS treatment caused a robust increase in IL-6 release from BV-2 cells in a concentration-dependent manner (data not shown). The EC₅₀ was selected based on the concentration-dependent curve of IL-6 release. Cells exposed only to the culture medium or to the medium supplemented with the nutrients alone, did not release any detectable amounts of IL-6 (data not shown). MIX A did not cause any significant modulation of the IL-6 release. MIX B decreased IL-6 release to 77% (IQR, 71-95.5) which was not statistically significantly different from untreated controls (U=8.000, Z=-0.816, p=0.994). In contrast, MIX C had a significant modulatory effect on LPS-induced IL-6 release (33.5%, IQR, 12.0-52.0, U=0.000, Z=-2.722, p=0.012).

The effects of MIX B and MIX C on IL-6 release did not differ from the effects observed for the corresponding most prominent single components in the mixtures (MIX B vs. GGPP: U=4.000, Z=-0.707, p=0.63; MIX C vs. FA: U=3.000, Z=-1.070, p=0.40).

3.2 Effects of decreasing concentrations of combinations of rice components on LPS-induced activation of BV-2 cells.

The lack of synergistic effects in the aforementioned experiments could be due to the relatively high concentrations of some components (GGPP, FA). Consequently, the anti-inflammatory effect of these individual nutrients may already be so strong that only a small window of opportunity remains for detecting a further decrease in inflammatory markers after combined treatment. To overcome this obstacle, we decreased the concentrations of FA, GGPP and GGOH to a level in which they did not decrease the NO release to more than 75% as compared to the LPS-stimulated
controls. Three different dilutions of each nutrient (starting from the highest nontoxic concentrations mentioned in Table 1) were tested. Based on the results depicted in Fig 2A-C, 3-fold dilutions of FA and GGPP and a 10-fold dilution of GGOH from the highest nontoxic concentration were selected for MIX D. As shown in figure 2D, rice components in these lower concentrations caused only a mild decrease in NO release (FA: 76%, IQR, 66.0-84.3%; GGOH: 86.5%, IQR, 80.0-91.0%, GGPP: 86.23, IQR, 81.0-91.8%). Combining these nutrients in the same low concentrations caused a further decrease in NO release to 65.9% (IQR, 61.0-71.4%), but this effect was not significantly different from the single nutrients, although mix D showed a trend towards increased efficacy as compared to GGOH or GGPP alone (GGOH vs. MIX D: U=0.000, Z=-2.309, p=0.058; GGPP vs. MIX D: U=0.000, Z=-2.323, p=0.058). Likewise, MIX D did not demonstrate any significantly better inhibition of IL-6 release than the single components (fig 2 E).

3.3 Effects of combinations of vitamins, fatty acids and rice bran components on LPS-induced activation of BV-2 cells.

To further investigate the effect of different combinations of nutrients on LPS-induced activation of BV-2 cells, we combined MIX D of the rice bran components with vitamins A and D and the fatty acids DHA and EPA. Combinations of these vitamins and fatty acids displayed enhanced anti-inflammatory effects in another study from our group (data will be published elsewhere). Several dilutions of the maximum nontoxic concentrations of the vitamins and fatty acids were tested. Based on the results in Fig 3 (A, C, E and G), 3-times dilution of the maximal nontoxic concentration for vitamin A and fatty acid DHA, 10-times dilution for vitamin D and no dilution for fatty acid EPA were selected for further experiments. As shown in figure 3 (B, D, F and H), combining vitamin A or DHA with MIX D did not cause any significant improvement in the inhibition of NO release. On the other hand, combining vitamin D with MIX D caused a trend towards enhanced inhibition of NO release (MIX D vs. MIX D + vitamin D: U=0.000, Z=-2.309, p=0.058; vitamin D vs. MIX D + vitamin D: U=0.000, Z=-2.323, p=0.058). Surprisingly, combining EPA with MIX D diminished the anti-inflammatory effect of MIX D. Although there was no statistical difference between EPA alone and the combination MIX D + EPA (U=6.500, Z=-1.180, p=0.257), a significant reduction of the efficacy of the combination of MIX D with EPA (to 84%, IQR, 73.0-93.0%) as compared to MIX D alone (U=1.000, Z=-2.374, p=0.038) was observed.
In summary, only the combination of vitamin D with \textit{MIX D}, which contains FA, GGPP and GGOH, showed a trend towards enhanced anti-inflammatory effect on NO release by LPS-stimulated BV-2 cells.

4. Discussion

The goal of the current study was to investigate the anti-inflammatory potency of different combinations of nutrients present in rice bran. We investigated combinations containing \(\gamma\text{OZ}, \text{FA}, \text{GGOH and GGPP}\). These substances, used separately, have already been shown to exert an anti-inflammatory effect on similar in-vitro models of neuroinflammation. In LPS-stimulated macrophages, \(\gamma\text{-OZ}\) inhibited the proinflammatory transcription factor NF-\(\kappa\)B, and consequently the production of proinflammatory enzymes such as COX-2 and iNOS, resulting in a reduction in the release of NO [12]. The phytosteryl ferulates from \(\gamma\text{OZ}\) have been demonstrated to inhibit NF-\(\kappa\)B activation in LPS-stimulated macrophages as well [25]. FA has been shown to inhibit NO, prostaglandin E2 and cytokine IL-1\(\beta\) release through inhibition of the proinflammatory enzymes iNOS and COX-2 in LPS-stimulated BV-2 cells [26]. Further research revealed that this anti-inflammatory effect is mediated by inhibition of NF-\(\kappa\)B and stimulation of Nrf2-mediated HO-1 [27]. The role of GGOH in the inflammatory response has been investigated on freshly isolated intraperitoneal macrophages. Insufficient endogenous production of GGOH resulted in an excessive inflammatory response to repetitive LPS stimulation, which manifested as a robust increase in LPS-stimulated release of the proinflammatory cytokines IL-1\(\beta\) and TNF\(\alpha\). The cytokine overproduction was reversed by exogenous administration of GGOH [28]. GGPP, to our knowledge, was not investigated before on LPS activated microglia or macrophages. However, GGPP was shown to inhibit statin-induced microglia activation [29]. Our experiments have shown that GGPP is also an effective inhibitor of LPS-induced NO release by BV-2 cells.

In the first part of the study, we investigated three combinations, \textit{MIX A}, \textit{MIX B} and \textit{MIX C}, containing \(\gamma\text{OZ}, \text{FA, GGOH and GGPP}\) in the same proportions as they occur in rice. We tested the efficacy of the mixes to attenuate NO release from LPS-stimulated BV-2 cells. NO is produced in activated immune cells from L-arginine. This reaction is catalysed by an enzyme, inducible NO synthase (\textit{iNOS}).
The expression of iNOS depends mainly on transcription factor NF-κB [30]. The ability to suppress NO release by activated immune cells is widely used as an indicator of anti-inflammatory potency. Evidence suggests detrimental effects can occur as a result of overproduction of NO, such as neurotoxicity [31]. Excessive NO production has been shown to occur in several brain pathologies, including neurodegenerative diseases [32]. Our experiments showed that MIX B and MIX C contain potent inhibitors of NO release in BV-2 cells. The anti-inflammatory effect of MIX B however was mainly due to the high concentration of GGPP, as MIX B has the same anti-inflammatory potency as the single component GGPP, when used in the same concentration. Similarly, the anti-inflammatory effect of MIX C was due to the effect of its main component, FA.

We also investigated the efficacy of MIX A, MIX B and MIX C to attenuate the release of cytokine, IL-6 from LPS-stimulated BV-2 cells. IL-6 is often used as a marker of LPS-induced microglia activation. Previous studies have demonstrated elevated serum levels of IL-6 following different noxious stimuli in animals and in humans. Moreover, increased levels of IL-6 have been correlated with disease severity and new treatment strategies for inflammatory diseases focusing on blocking IL-6 signalling are being developed [33,34]. MIX A and MIX B were not sufficiently effective to modulate LPS-induced IL-6 release from BV-2 cells. On the other hand, MIX C could significantly inhibit IL-6 release, but the anti-inflammatory effect of MIX C was attributed to the effect of its main component, FA.

In the second part of the study, we investigated MIX D containing the three most effective components, FA, GGOH and GGPP in sub-effective concentrations. Since FA and GGOH had a strong anti-inflammatory effect when used at the high concentrations in MIX B and MIX C, the window of opportunity to detect the additive effect of combining them with other nutrients was very small. Our previous studies demonstrated that using a lower concentration of nutrients can allow detection of synergistic and additive anti-inflammatory properties due to the increased detection window [24]. However, in the present study, mixing FA, GGOH and GGPP at sub-effective concentrations (MIX D) did not result in a synergistic reduction in NO release compared to the effect caused by single components.
Taken together, our results demonstrated dose-dependent anti-inflammatory properties of phytochemicals present in rice bran, but mixing the ingredients in the same proportions as they occur in rice did not enhance their anti-inflammatory efficacy in vitro. This could be due to a similar mode of action of the investigated nutrients. γOZ and FA were both described to inhibit the transcription factor NF-κB. The mechanism of action of GGOH and GGPP has not yet been elucidated, although it is known that both substances are involved in the mevalonate synthesis.

Furthermore, we investigated whether combining MIX D with other nutrients could enhance their anti-inflammatory effect. We selected vitamins A and D, and fatty acids DHA and EPA in concentrations that showed enhanced anti-inflammatory effects on LPS-stimulated BV-2 cells, when mixed [24]. Our current experiments demonstrated a trend towards enhanced anti-inflammatory efficacy, when combining vitamin D with the MIX D. This could be due to the synergy between independent pathways, since vitamin D interacts not only with NF-κB, but also with vitamin D receptors (VDR) [35]. Recent findings demonstrated a mechanism of inhibition of LPS-induced NO release from BV-2 cells by vitamin D, which is mediated by VDR [36].

In conclusion, this study shows a possible convergence between food components interacting with diverse pathways, which is a promising concept to be tested in future studies.

**Figures and tables**

**Table 1** Concentrations of nutrients used in mixtures investigated in the first part of the study. The maximum nontoxic concentration was assessed with a viability test. The substances were mixed in the same proportions as they occur in rice bran, in such a way that the maximum tolerable concentration will not be exceeded. (on the next page)
<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Abbreviation</th>
<th>µg/g rice</th>
<th>Max. conc. based on the viability test</th>
<th>Mix A µg/ml</th>
<th>Mix B µg/ml</th>
<th>Mix C µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gamma-oryzanol</td>
<td>γOZ</td>
<td>1740</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td></td>
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<tr>
<td>Geranylgeranyl pyrophosphate ammonium salt</td>
<td>GGPP</td>
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<td>9.0</td>
<td>0.00414</td>
<td>8.63</td>
<td></td>
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<tr>
<td>Trans-ferulic acid</td>
<td>FA</td>
<td>20</td>
<td>192</td>
<td>0.02874</td>
<td>59.93</td>
<td>192</td>
</tr>
<tr>
<td>geranylgeraniol</td>
<td>GGOH</td>
<td>0.09</td>
<td>1.74</td>
<td>0.00013</td>
<td>0.27</td>
<td>0.864</td>
</tr>
</tbody>
</table>

**Figure 1** The effects of different combinations of nutrients on LPS-induced NO and IL-6 release. Concentrations and combinations of nutrients listed in table 1 (MIX A, B, C) and their 10-fold and 100-fold dilutions, indicated as 0.1x and 0.01x, respectively, were used. D, E the concentration of GGPP was the same as in MIX B and the concentration of FA was the same as in MIX C. BV-2 cells were pre-treated for 24 h with different combinations of 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-D) and IL-6 release (E-H) with ELISA. 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 and IL-6 for LPS control (100%) is indicated on each graph with the dashed line. All graphs represent the results from four independent experiments (median ±SEM). Data were statistically analysed with Kruskal-Wallis H test and a difference was considered statistically significant when p < 0.05 (outcome values from that test are mentioned on upper right part of each graph). To test for the individual differences between the groups, a Bonferroni-Holm post-hoc test was
applied. A difference was considered statistically significant when $p < 0.05$ and indicated as significantly different as compared to the LPS control with (*).
Figure 2 The effects of different concentrations of nutrients and their mixture in sub-effective concentrations on LPS-induced NO. First, nutrients were used in highest nontoxic concentrations: FA - 192 µg /ml, GGOH – 1.74 µg /ml, GGPP – 9.0 µg /ml. In addition, the nutrients were tested when diluted 3 times (indicated as 0.33x) and 10 times (indicated as 0.1x) (A-C). Furthermore, the sub-effective concentrations of each nutrient were combined in MIXD (D-E). BV-2 cells were pre-treated 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 or IL6 release by ELISA. NO and IL6 levels are presented as the percentage of the concentration produced by cells treated with LPS alone (LPS control). The level of NO or IL6 for LPS control (100%) is indicated on each graph with the dashed line. All graphs represent the results from 4 independent experiments (median ±SEM). To test for the individual differences between each nutrient and LPS, a Mann Whitney U test was applied for each comparison. A difference was considered statistically significant when p < 0.05 and indicated as significantly different as compared to the LPS control with (*). (on the next page)
Figure 3 The effects of different combinations of nutrients on LPS-induced NO release after decreasing the nutrient concentrations and adding vitamins or fatty acids. Nutrients were used in highest non-toxic concentrations: vitamin A - 1.75 µM, vitamin D - 1 µg/ml, DHA, EPA - 20 µM (indicated as 1x); further the nutrients were diluted 3 times (indicated as 0.33x) and 10 times (indicated as 0.1x). MIX D indicates the combination of FA, GGOH and GGPP in the concentrations chosen based on the data from fig 2. BV-2 cells were pretreated for 24 h with different combinations of 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. NO levels are presented as the percentage of the concentration produced by cells treated with LPS alone (LPS control). The level of NO for LPS control (100%) is indicated on each graph with the dashed line. All graphs represent the results from four independent experiments (median ±SEM). Data were statistically analysed with Kruskal-Wallis H test. A difference was considered statistically significant when p < 0.05 (outcome values from that test are mentioned on upper right part of each graph). To test for the difference between the treatment conditions, a Bonferroni-Holm post-hoc test was applied. A difference was considered statistically significant when p<0.05 and indicated as significantly different as compared to the LPS control with (*). (on the next page)
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Chapter 4


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