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Spirulina diet to lactating mothers protects the anti-oxidant system and reduces inflammation in post-natal brain after systemic inflammation

Jaspal Patil¹, Ashok Matte¹, Carina Mallard², and Mats Sandberg¹

¹Department of Medical Biochemistry and Cell Biology, Institute of Biomedicine, University of Gothenburg, Sweden

²Department of Physiology, Institute of Neuroscience and Physiology, University of Gothenburg, Sweden

Abstract

Objectives—This study concerns: 1) the long-term effects of peripheral LPS in neonatal rats on inflammation and anti-oxidant parameters in brain and 2) the effects of a *Spirulina* enriched diet given to lactating mothers on protective and inflammatory parameters in brains of suckling pups subjected to peripheral inflammation.

Methods—Five-day old rat pups were treated with LPS (i.p. 2 mg/kg). After 3, 7, 30 and 65 days, mRNA, miRNA and protein levels of pro-inflammatory cytokines and the Nuclear factor E2-related factor 2 (Nrf2)-system were examined. In a sub-group a *Spirulina* enriched diet was given to the mothers 24 h before the pups were treated with LPS, then the effects on anti-oxidant and inflammatory parameters were evaluated.

Results—The main findings were: 1) IL-1 β was upregulated in cortex 3, 7 and 30 days after LPS treatment, 2) Nrf2 and the catalytic subunit of γ -glutamylcysteinyl ligase were decreased in cortex 7 days after LPS in parallel with increased levels of phosphorylated p38 and decreased levels of histone H3 acetylation and 3) a *Spirulina* enriched diet to lactating mothers normalized both the increased IL-1 β expression and the decreased anti-oxidant parameters after LPS. The protective effects of *Spirulina* were correlated with decreased levels of phosphorylated p38 and high levels of the anti-oxidant miRNA 146a.

Discussion—A *Spirulina* diet given to lactating mothers can protect against neuroinflammation and decreased anti-oxidant defence in brain of suckling pups subjected to peripheral inflammation, possibly via decreased activation of p38 and high levels of the anti-oxidant miRNA 146a.

Keywords

neuroinflammation; Nrf2; antioxidant system; *Spirulina*; miRNA

1. Introduction

Severe bacterial infection in the perinatal period has been estimated to account for a global burden of disease similar to that of HIV/AIDS. Most of the burden is related to neonatal deaths, but survivors are also at risk for long-term disability [1]. Inflammation in the neonatal period can lead to dysfunctional brain development [2, 3] and for example, maternal infection during pregnancy is associated with an increased incidence of cerebral palsy and neurological damage in preterm infants [4–6]. Experimental studies show that mental or behavioral disorders in the off spring correlate with the stimulation of the maternal immune system [7], and prenatal injection of endotoxin (lipopolysaccharide (LPS)) can cause increased anxiety, decreased social interactions [8, 9] and memory impairments [10, 11] in rats.

Even if the bacterial infection *per se* can be eradicated the infection may have initiated mechanisms that cause long term effects. Experimental studies show that perinatal infection can increase vulnerability to a secondary excitotoxic challenge [12] and to hypoxic-ischemic injury [13, 14]. However, depending on the circumstances perinatal systemic inflammation can also have a pre-conditional effect and protect against hypoxic-ischemic injury [15]. Thus, identification of the mechanisms behind the long-term effects of a neonatal infection is an important step towards effective treatment in humans. Numerous experimental models have been developed to investigate the effects of peripheral inflammation on brain function and protection [16]. Among the putative mechanisms from perinatal infection to an altered molecular phenotype in adolescence/adulthood are changes in expression of pro-inflammatory/anti-inflammatory cytokines, in trophic factors and in defense systems to oxidative stress. One important factor concerning regulation of both inflammation and oxidative stress is the transcription factor Nuclear factor E2-related factor 2 (Nrf2). Activation of Nrf2 by endogenous oxidative stress, exogenous oxidative substances or other factors that activate key regulatory kinases causes increased transcription of hundreds of protective genes and decreased activation of pro-inflammatory cytokines [17]. On the other hand, deletion of the Nrf2 gene renders brains of animals hypersensitive to neuroinflammation and more sensitive to challenges such as oxygen deprivation and intracerebral hemorrhage [17–19].

We have earlier shown that LPS-mediated inflammation *in vitro* and *in vivo* can cause both up-regulation (after 24 h) and down-regulation of Nrf2 protein (after 72 h) [20]. In the present study the objectives were to evaluate if perinatal inflammation can lead to more prolonged effects in the Nrf2-system and if a diet enriched in extracts of the blue-green algae *Spirulina Platensis* could counteract putative negative effects. This edible algae contains antioxidant and anti-inflammatory phytochemicals such as carotenoids, c-phycocyanin and c-phycocyanobilin. Extracts of *Spirulina* or isolated c-phycocyanin and c-phycocyanobilin have potent antioxidant and anti-inflammatory activity [21, 22]. A diet enriched in *Spirulina* can enhance the recovery of dopamine neurons in an animal model of Parkinson's disease [23]. The study showed that animals fed with the *Spirulina* diet had reduced lesion and decreased microglia activation although the diet allowed for a robust early microglial response [23]. A diet supplemented with *Spirulina* has also been shown to reverse an inflammation induced decrease in stem/progenitor cell proliferation in brain [24].

We employed a well-established model where systemic LPS in PND 5 rats have been shown to increase the vulnerability to a neurotoxin 65 days after the initial endotoxin exposure [25]. The mRNA and protein levels of the Nrf2 defense system and pro-inflammatory cytokines in neocortex and the hippocampus were analyzed 3, 7, 30 and 65 days after LPS. The effects of a diet supplemented with 0.1% *Spirulina* (SP0.1%) extract, given to the lactating mothers, were evaluated 7 days following the LPS treatment.

2. Material and Methods

2.1 Animal Treatment and Tissue Preparation

Sprague-Dawley rats from our in-house colony were housed in a 12-hour light-dark cycle and bred at Experimental Biomedicine, University of Gothenburg, Sweden with free access to food and drinking water. Animal experiments were approved by the Ethical Committee of Gothenburg (No. 264-2009 and 205-2012) and followed the guidelines for the care and use of experimental animals and the European Communities Council Directive of 24 November 1986 (86/609/EEC). At postnatal day (PND) 5 rat pups were injected intraperitoneally (i.p.) with saline or LPS (2 mg/kg) dissolved in saline (LPS isolated from *E. coli* 055:B5, List Biological Laboratories, INC, Campbell, CA, USA). Cerebral cortex and hippocampus were dissected and collected at 3, 7, 30 and 65 days following LPS injection (Fig 1A). In one set of experiments rat dams were fed control diet or the same diet containing SP0.1% (Earthrise Nutritionals, Irvine, CA, USA), beginning 24 h prior to the LPS injection of the pups (Fig 1B). The rat pups were sacrificed 3 and 7 days after LPS by an overdose of thiopental sodium or after 30 and 65 days by isoflurane anesthesia (Baxter Medical AB, Sweden) followed by decapitation. The different brain regions were collected and immediately frozen in liquid nitrogen, and stored at -80°C .

2.2 Quantitative real time PCR

Cerebral cortex and hippocampus collected following saline or LPS injection ($n = 11-16$, per group, from at least 8 mixed litters) were used for total RNA extraction by using RNeasy Lipid Tissue Mini Kit (Qiagen, Solna, Sweden). Total RNA concentration was measured in a spectrophotometer at 260-nm absorbance (NanoDrop 2000/2000c, Thermo Scientific). 1 μg of total RNA from each sample was used for first strand cDNA synthesis according to the manufacturer's instructions (QuantiTect Rev. Transcription Kit, Qiagen, Solna, Sweden). For miRNA extraction miRNeasy Mini Kit (Qiagen, Solna, Sweden) was used; 1 μg of miRNA was used for first strand cDNA synthesis by using miScript II RT Kit (Qiagen, Solna, Sweden). Then miRNA expression was analyzed by using miScript SYBR Green PCR Kit (Qiagen, Solna, Sweden). For miRNA quantitative real time PCR (qRT-PCR) each reaction (20 μl) contained 20 ng of cDNA in 5 μl volume, 10 μl miScript SYBR Green PCR Kit (Qiagen, Solna, Sweden), 2 μl PCR primers, 2 μl of miScript Universal Primer and 1 μl of H_2O to make final reaction volume of 20 μl . In order to determine mRNA expression the cDNA samples were further processed by qRT-PCR. Each PCR reaction (20 μl) contained 20 ng of cDNA in 5 μl volume, 10 μl Quanti Fast SYBR Green PCR Master Mix (Qiagen, Solna, Sweden), 2 μl PCR primers and 3 μl of H_2O to make a final reaction volume of 20 μl . The lists of primers used for qRT-PCR are provided in Table 1 (Qiagen, Solna, Sweden).

The amplification protocol comprised an initial 5 min denaturation at 95 °C, followed by 40 cycles of denaturation for 10 s at 95 °C and annealing/extension for 30 s at 60 °C on a LightCycler 480 (Roche, Sweden). Melting curve analysis was performed to ensure that only one PCR product was obtained. For quantification and estimation of amplification efficiency, a standard curve was created using increasing concentrations of cDNA. The amplification transcripts were quantified with the relative standard curve and normalized against the geomean of reference genes beta-glucuronidase (GUSB), hypoxanthine-guanine phosphoribosyltransferase (HPRT1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), while miRNA-quantification results were normalized to an internal control miR-16, an ubiquitously produced microRNA.

2.3 Western Blot Analysis

2.3.1 Sample preparation—The protein fraction obtained from the silica column (first flow through) in the RNA extraction steps was used for acetone precipitation of proteins as described by the manufacturer. Protein concentration was determined by the bicinchoninic acid assay (BCA assay). Protein homogenates were mixed with 1: 3 (v/v) Laemmli sample buffer (Bio-Rad, Hercules, Calif., USA) plus 5% (w/v) β -mercaptoethanol and boiled for 5 min. An equal amount of protein (20 μ g) was resolved on 8–16% Criterion™ TGX Stain-Free™ Gel (Bio-Rad, Hercules, Calif., USA) and electrophoresis was done by using 10 $\times$ Tris/Glycine/SDS premixed electrophoresis buffer (Bio-Rad, Hercules, Calif., USA) following dilution to 1 $\times$ concentration. Gels were run at 300 V for 21 min. For blotting, gels were further processed by using Trans-Blot® Turbo™ Midi Nitrocellulose Transfer Packs (Bio-Rad, Hercules, Calif., USA) and electroblotted at 25 V for 7 min. The membranes were blocked for 1 h at room temperature in 5% (w/v) dry skimmed milk (Semper Mjöl, Sundbyberg, Sweden) in TBS with 0.1% Tween 20 (TBST). The membranes were then incubated overnight at 4 °C with the corresponding primary antibodies in 5% bovine serum albumin-TBST, thoroughly washed with TBST solution and incubated with the correspondent secondary antibodies for 1 h at room temperature. Lastly, the blots were rinsed with TBST and the peroxidase reaction was developed by enhanced chemiluminescence SuperSignal® West Dura Extended Duration Substrate (Thermo Scientific, Rockford, Ill., USA). Blots were stripped in stripping Buffer (Tris base 62.7 mM, SDS 69.5 mM, β -mercaptoethanol 111.6 mM, pH 6.7) and were reused sequentially. Images were captured with a Fujifilm Image Reader LAS-1000 Pro version 2.6 (Fujifilm, Stockholm, Sweden), and the different band intensities (density arbitrary units) corresponding to immunoblot detection of protein samples were quantified using the Fujifilm Multi Gauge version 3.0 software (Fujifilm, Stockholm, Sweden).

2.3.2 Antibodies—Rabbit-anti-acetyl-histone H3 (1:1000, Millipore, Solna, Sweden), mouse-anti-Nrf2 (1:500, R&D Diagnostics, Minneapolis, Minn., USA), rabbit-anti pSer9GSK3 β (1:1000, Cell Signaling, Beverly, USA), rabbit anti-GSK3 β (1:1000, Cell Signaling, Beverly, USA), rabbit-anti-phosphorylated p38 (1:500), rabbit-anti-p38 (1:500), mouse-anti- α -tubulin (1:2000), rabbit-anti- γ -glutamylcysteine ligase catalytic subunit (γ GCLC) (1:500) and rabbit-anti- γ -glutamylcysteine ligase modulatory subunit (γ GCLM) (1:500) antibodies were from Santa Cruz Biotechnology, Heidelberg, Germany. Peroxidase-

conjugated anti-rabbit (1:5000) and anti-mouse (1:5000) secondary antibodies were from Vector Laboratories (Burlingame, Calif., USA).

2.4 Determination of IL-1 β protein by ELISA

IL-1 β protein levels were determined by DuoSet ELISA Development kit (R&D systems, Minneapolis, Minn., USA #DY501), following the manufacturer's instructions and data were acquired using a 96-well plate reader (SpectraMax® Plus, Molecular devices, Sunnyvale, CA, USA).

2.5 Statistical Analysis

Results are presented as means $\pm$ standard error of the mean (SEM). The Mann–Whitney U test was used to determine statistical significance (95%; $p < 0.05$) between two groups. For analysis of the interaction of diet on LPS treatment two-way ANOVA analysis were performed by the SAS PROC MIXED using the SAS v.9.3 (SAS Inc., Cary, N.C., USA), followed by Bonferroni post-hoc test. Significance levels were at * p 0.05, ** p 0.01, *** p 0.001. The Mann-Whitney and the Bonferroni tests were performed by using GraphPad Software.

3. Results

The pups injected with LPS (i.p.) showed an average of 10.5% to 8.8% reduction in weight gain on PND 6, 7, 8 and 9 (Supplementary data Fig. S1A). No difference was observed in the increase of interleukin-1 β (IL-1 β) mRNA in the brain between male and female rat brain 7 days following the LPS injection (Supplementary data Fig. S1B).

3.1 Time course of the effects of systemic LPS

The level of IL-1 β mRNA in cerebral cortex (Fig. 2A) was significantly increased 3, 7 and 30 days after LPS treatment while tumor necrosis factor- α (TNF α) mRNA (Fig. 2B) remained at control levels at all-time points. Similar stimulatory effects of LPS on IL-1 β mRNA levels 3, 7 and 30 days after LPS were observed in hippocampus (Fig. 2C), where also TNF α mRNA levels were upregulated after 3 and 7 days in hippocampus (Fig. 2D). Parts of the Nrf2-system were increased (Nrf2 and Heme oxygenase 1 (HO-1)) 3 days after LPS in both cortex and hippocampus (Fig. 3; Fig. S2) whereas reduced levels were observed only in the cortex for the subunits in the rate limiting enzyme of glutathione synthesis 7 and 30 days following LPS (γ GCLM, γ GCLC (Fig. 3; Fig. S2). The mRNA level of PGC-1 α was decreased in cortex both 3 and 7 days after LPS treatment (Fig. 3). All LPS-induced changes were restored in 70 day old rats (Fig. 3).

3.2 A Spirulina enriched diet decreased LPS induced IL-1 β upregulation

The levels of mRNA of IL-1 β (Fig. 4A) as well as IL-1 β protein (Fig. 4B) were increased in the cortex 7 days after LPS injection in pups from dams fed a standard diet. Feeding the dams with a diet supplemented with SP0.1% 24 h before the pups were injected with LPS restored the level of IL-1 β mRNA (Fig. 4A). No significant interaction between diet and the LPS treatment was observed for IL-1 β protein. However, a significant decrease in the level

of IL-1 β mRNA and protein in cerebral cortex was observed in LPS injected pups from the litters where dams had been given a diet supplemented with SP0.1% (Fig. 4A & B).

3.3 A Spirulina enriched diet normalized LPS induced effects on the Nrf2 system

The mRNA levels of Nrf2 (Fig. 5A) and its down-stream genes γ GCLC (Fig. 5B) and γ GCLM (Fig. 5C) were decreased 7 days following LPS injection. These effects were significantly normalized by the SP0.1% enriched diet given to the dam. A similar trend was observed for PGC-1 α mRNA (Fig. 5D). The corresponding protein levels of γ GCLC (Fig. 6C), was also significantly reduced by the LPS treatment and restored by the maternal SP0.1% diet. No significant interaction between diet and the LPS treatment was observed for Nrf2 protein. However, a significant increase in the level of Nrf2 protein in cerebral cortex of LPS injected animals was observed when the dams had been given a diet supplemented with SP0.1% (Fig. 6B).

3.4 The LPS induced changes in the Nrf2 regulatory kinase p38 and histone H3 acetylation in cortex of pup brains were partly normalized by a Spirulina enriched diet given to the mother

Active phosphorylated p38 (pp38) was increased in cerebral cortex 7 days following systemic LPS (Fig. 7A) while no increases were observed 3, 30 and 65 days after LPS injection (Fig S3). The levels of phosphorylated GSK-3 β were not altered 3, 7, 30 or 65 days after the LPS injection (Fig. S3). The level of acetylated histone H3 was decreased 7 days after LPS while no effects on acetylated histone H3 were observed after 3, 30 and 65 days (Fig. S3).

A statistically significant interaction between diet and LPS treatment was observed for acetylated histone3, but not for pp38 (Fig. 7). However, a significant decreased level of pp38 protein in cerebral cortex of LPS injected animals was observed when the dams had been given a diet supplemented with SP0.1% (Fig. 7C)

3.5 The LPS induced increase in the miRNA146a in cortex of pup brains was reduced by a Spirulina enriched diet given to the lactating mothers

The level of inflammation and activation of the Nrf2-system can be regulated by various miRNAs. Here we show that miR-146a, a regulator of inflammation [26] was significantly elevated 3, 7 and 30 days following LPS, but restored in PND 70 animals after LPS treatment (Fig. 8A). Expression of miR-146a was significantly increased in cortex at 7 days after LPS compared to saline in pups with a dam on standard diet. In pups, where the mothers were fed with a diet enriched with SP0.1%, the expression of miR-146a was significantly increased compared to saline treated pups, but was significantly reduced compared to LPS-treated pups from mothers on standard feed (Fig. 8B). The levels of other miRNAs (miRNA-153, miRNA-200a, miRNA-142, miRNA-144, miRNA-27a, miRNA-28, and miRNA-93* and precursor miRNA-93, for primer information see supplementary Table 1) that have been shown to be linked to Nrf2 regulation, did not change 7 days following the LPS injection (Fig. S5).

4. Discussion

4.1 Prolonged effects of neonatal LPS on IL-1 β and Nrf2

Here we confirm that systemic LPS in PND5 rats result in prolonged neuroinflammation as measured by increased protein levels and mRNA of IL-1 β in the brain. An intriguing finding is the prolonged increase in cortical IL-1 β expression but not in TNF- α [25]. The reason for this specific effect may relate to epigenetic modification of the promoter which recently was shown as a mechanism for selective up-regulation of IL-1 β expression [27]. The early increase and delayed decrease of Nrf2 by inflammation is in accordance with our earlier studies [20, 28, 29]. In the earlier *in vivo* study (0.3 mg LPS/kg i.p.) we found increase in Nrf2 after 24 h and a down-regulation 3 days following the LPS injection [20]. In the present study (2 mg LPS/kg i.p.) the expression of the Nrf2 mRNA was still increased 3 days after the LPS systemic injection, decreased by 7 days and normalized 30 days after LPS. The temporal shift is likely caused by a prolonged acute phase of inflammation as a result of the higher dose of LPS.

4.2 Elevated activated p38 and reduced acetylated histone H3 7 days following neonatal LPS

In our earlier studies *in vitro* we found that an inflammation mediated decrease in Nrf2-mediated transcription correlated with increased levels of the activated forms of p38 and GSK-3 β as well as with decreased levels of acetylated histone H3 [28]. Here we found that active p38 was increased and acetylated histone H3 was decreased 7 days following LPS injection but not at 3, 30 or 65 days following the LPS injection (Fig. S3). No effects of LPS on the phosphorylated level of GSK-3 β were observed at any time point (Fig. S3). Nrf2 expression is down-regulated in the course of brain development [30] via hypoacetylation of histone H3 which supports that the histone H3 hypoacetylation observed here indeed can be the mechanism behind the Nrf2 decrease 7 days following the LPS injection. Whether the decrease in histone H3 acetylation is related to the elevated level of active p38, as we showed earlier *in vitro*, remains to be evaluated *in vivo*.

4.3 Decreased levels of subunits of the rate limiting enzyme in glutathione synthesis 7 and 35 after neonatal LPS

Interestingly the mRNA levels of γ GCLC and γ GCLM, coding for the subunits of the rate limiting enzymes in glutathione synthesis, were still suppressed even 30 days following the injection of LPS (Fig. 3) whereas mRNA of another Nrf2 target gene, HO-1, was normal (Fig. 3). This indicates different mechanisms behind the decreased Nrf2 levels (only observed after 7 days following LPS) and γ GCLC/ γ GCLM (observed both after 7 and 30 days following LPS) expression. A malfunctioning Nrf2-GCLC system has been suggested as a mechanism behind diabetic retinopathy [31]. The mechanism causing the decreased Nrf2 transcriptional efficacy was related to aberrant methylation of lysine 4 in histone H3 (H3K4) [32]. Of high interest concerning the selective decrease in γ GCLC and γ GCLM mRNA is the finding that lung cells subjected to smoke show a persistent selective decrease in expression of γ GCLC that correlated to increased DNA methylation level of the γ GCLC gene promoter. No significant differences in DNA methylation of the promoters of other Nrf2-targets were observed [33]. Thus our working hypothesis is that the decrease in Nrf2-

expression 7 days following LPS is related to the high level of active p38 and low acetylated histone H3 levels and that the selective decreases in γ GCLC/ γ GCLM mRNA expressions 30 days after LPS may relate to increased promoter methylation or histone methylation as described above.

4.4. A diet enriched in *Spirulina* to lactating mothers reduced the LPS induced neuroinflammation and restored the antioxidant defense

Enrichment of a diet with *Spirulina* given to adult animals has earlier been shown to be neuroprotective, for example age induced increases in pro-inflammatory cytokines are reduced, neurodegeneration after stroke is decreased and substantia nigra neurons in models of Parkinson's disease are protected [23, 24, 34]. A few studies have shown that a *Spirulina* supplement given to the pregnant mother can have neuroprotective effects in the offspring [35]. As far as we know our study is the first to show that a *Spirulina* enriched diet given to the lactating mothers have anti-inflammatory and restoring effects on defense systems in brains of neonatal rats subjected to systemic inflammation. The normalizing effects by *Spirulina* on IL-1 β , Nrf2 and γ GCLC levels in our study can be exerted by different mechanisms. The protective effects *in vivo* are generally attributed to the anti-oxidant and free radical scavenging properties of the active ingredient C-phycocyanin [36]. Concerning mechanistic studies *in vitro* *Spirulina* components have also been shown to reactivate the kinase Akt [37], [38] decrease the expression of HDACs, restore LPS mediated decreases in acetylated levels of histone H3 [39], down-regulate pp38 and upregulate ERK1/2 [40]. In our earlier studies *in vitro* we showed that active p38 had negative and that ERK1/2 had positive effects of Nrf2 activation [28]. Thus, our results *in vivo* showing that a *Spirulina* diet normalized the LPS mediated increases in pp38 and the decreases in the acetylated H3 agree well with effects of *Spirulina* in the above mentioned *in vitro* studies.

4.5 The level of anti-inflammatory miRNA-146a was increased by neonatal LPS and was still elevated in brains of rats when the lactating mother was given a *Spirulina* enriched diet

The miRNA 146a levels were upregulated 3, 7 and 30 days following LPS treatment of PND 5 rats, similar to the increased levels of IL-1 β mRNA. Other miRNA of relevance for Nrf2 expression were unchanged (Fig. S5). The miRNA 146a has been shown to have anti-inflammatory activity via reduced levels of the miR-146a targets IL-1R-associated kinase 1 (Irak1) and TNFR-associated factor 6 [41]. However, we found no decrease in interleukin-1 Receptor-Associated Kinase (IRAK) 2 [42], FAS [43], IRAK1 or TNF receptor-associated factor 6 (TRAF6) (Fig. S4). It has also been shown that IL-1 β -induced increases in miRNA-146a expression can negatively regulate chemokine release [44]. Interestingly, the level of miR-146a in cortex was reduced but still significantly increased in brains of pups treated with LPS whose mothers had been given the SP0.1% diet. In contrast the levels of IL-1 β mRNA and protein were not significantly increased 7 days following LPS treatment in brains of pups from mothers given SP0.1% diet. Thus, the *Spirulina* diet to lactating mothers preserved the LPS-induced increase in anti-inflammatory miR146a, and decreased the pro-inflammatory IL-1 β in pup brains.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

BCA	Bicinchoninic acid assay
COPD	Chronic obstructive pulmonary disease
ERK1/2	extracellular regulated kinase
γGCLC	gamma-glutamylcysteine ligase catalytic subunit
γGCLM	gamma-glutamylcysteine ligase modulatory subunit
GCS	γ-glutamylcysteine synthetase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GSK3β	glycogen synthase kinase-3 beta
Gusb	beta-glucuronidase
GSH	glutathione
HDAC	Histone deacetylases
HO-1	Heme oxygenase 1
HPRT1	hypoxanthine-guanine phosphoribosyltransferase
IL-1β	Interleukin 1 beta
IRAK1 and IRAK2	Interleukin-1 Receptor-Associated Kinase 1 and 2
Keap1	Kelch-like ECH-associated protein 1
LPS	lipopolysaccharide
MAPKs	mitogen-activated protein kinases
Nrf2	nuclear factor-erythroid 2-related factor 2
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator-1 alpha
SP0.1%	Spirulina 0.1%

TRAF6

TNF receptor-associated factor 6

Tnfa

tumor necrosis factor-alpha

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Summary

Our study shows that neonatal systemic inflammation can cause prolonged decreases in anti-oxidant defense systems and elevated levels in the pro-inflammatory cytokine IL-1 β as well as the anti-inflammatory miRNA-146a. The implication for humans is that a neonatal severe infection can cause long-term increased vulnerability to oxidative stress in the neocortex even after the sepsis/infection has been treated. As putative mechanisms behind the negative effects on the Nrf2-system we identified elevated levels of active p38 and decreased levels of acetylated histone H3. A Spirulina enriched diet given to lactating mothers reduced the prolonged neuroinflammation and restored the anti-oxidant defence in the neonatal rat brain after LPS possibly via normalizing the levels of active p38 and restoring the acetylation levels of histone H3 in combination with a preserved increase in the anti-inflammatory miR-146a. Thus, additions to the mother's diet of phytochemicals that can counteract disease mechanisms on various levels [45] can be an attractive way to reduce the prolonged neuroinflammation and negative effects on the anti-oxidant system induced by neonatal systemic inflammation.

Figure 1.

Schematic outline of the experiments. Rat pups were treated with saline or LPS at post-natal day (PND) 5 and sacrificed at PND8, PND12, PND35 or PND70 (Fig. 1A). In a separate set of experiments lactating mothers were provided with a diet containing 0.1% *Spirulina* extract (SP0.1%) 24 h before LPS treatment of the pups at PND5 (Fig. 1B). The pups in these experiments were sacrificed on PND12.

Figure 2.

Effects of LPS (PND5) on expression of IL-1 β and TNF α mRNA in cerebral cortex and hippocampus at PND8, PND12, PND35 and PND70 (see Fig. 1A and 2.1-2.2 for details). Quantitative real time-PCR analysis was performed for IL-1 β (Fig. 2A) and TNF α (Fig. 2B) mRNA in cerebral cortex and IL-1 β (Fig. 2C) and TNF α (Fig. 2D) mRNA in hippocampus. Data is expressed as relative expression of IL-1 β and TNF α mRNA normalized to the geometric mean of housekeeping mRNAs GAPDH, GUSB and HPRT1. Values are expressed as fold change over control, $n = 8-10 \pm \text{SEM}$. * Indicates significant differences ($p < 0.05$), compared to control (saline injected pups) using the Mann–Whitney U test.

Figure 3.

Effects of LPS (PND5) on expression of the Nrf2-system in cerebral cortex at PND8, PND12, PND35 and PND70 (see Fig. 1A and 2.1-2.2 for details). Quantitative real time-PCR analysis was performed for Nrf2 (Fig. 3A), Keap1 (Fig. 3B), HO1 (Fig. 3C), γ GCLC (Fig. 3D), γ GCLM (Fig. 3E) and PGC1 α (Fig. 3F) mRNA. Data represent relative expression of Nrf2-system genes normalized to geometric mean of GAPDH, GUSB and HPRT1 mRNA. Values are expressed as fold change over control ($n = 8-10 \pm \text{SEM}$).

*Indicates significant differences ($p < 0.05$) compared to control (saline injected pups) using the Mann-Whitney U test.

Figure 4.

Effects of maternal SP0.1% diet on LPS (PND5) induced changes in IL-1 β in the pup cerebral cortex (PND12) (see Fig. 1A-B and 2.1-2.3 for details). Quantitative real time-PCR analysis and ELISA were performed for determination of IL-1 β mRNA and protein at PND12. For IL-1 β mRNA, data represents relative expression normalized to geometric mean of GAPDH, GUSB and HPRT1 mRNA. For IL-1 β protein, two-way ANOVA showed a statistically significant interaction between diet and LPS treatment, $p = 0.0062$ and Bonferroni post-hoc analysis showed significant differences for Saline ($n = 16$) vs LPS ($n = 15$) and LPS vs LPS+SP0.1% ($n = 14$). For IL-1 β protein, two-way ANOVA did not show a significant interaction between diet and treatment for IL-1 β protein, but did reveal main effects of diet and LPS treatment and Bonferroni post-hoc analysis for IL-1 β protein data showed significant differences for Saline ($n = 9$) vs LPS ($n = 8$) and LPS vs LPS+SP0.1% ($n = 14$). *denotes statistical significance ** $p < 0.01$; *** $p < 0.001$). Values are expressed as means $\pm$ SEM.

Figure 5.

Effects of maternal SP0.1% diet on LPS (PND5) induced changes in the Nrf2-system in pup cerebral cortex (PND12) (see Fig. 1A-B and 2.1-2.2 for details). Quantitative real time-PCR analysis was performed for Nrf2 (Fig. 5A), γGCLC (Fig. 5B), γGCLM (Fig. 5C) and PGC1α (Fig. 5D). Data are expressed as relative expression normalized to geometric mean of GAPDH, GUSB and HPRT1 mRNA. Two-way ANOVA showed a statistically significant interaction between diet and LPS treatment for: Nrf2, $p=0.0167$; GCLC, $p=0.0013$; GCLM, $p=0.0019$; PGC1α, $p=0.0039$. Bonferroni post-hoc analysis comparing Saline ($n=16$) vs LPS ($n=15$) and LPS vs LPS+SP0.1% ($n=14$) groups are shown, *denotes statistical significance (* $p<0.05$; ** $p<0.01$; *** $p<0.001$; ns-not significant). Values are expressed as means $\pm$ SEM.

Figure 6.

Effect of maternal SP0.1% diet on LPS (PND5) induced changes in Nrf2 and γ GCLC protein expression in the pup cerebral cortex (PND12), (see Fig. 1A-B and 2.1-2.3 for details). Protein expression levels of Nrf2 and γ GCLC are shown in Fig. 6A; the respective densitometric analyses of Western blot are depicted in Fig. 6B and Fig. 6C. Two-way ANOVA showed a statistically significant interaction between diet and LPS treatment for GCLC, $p = 0.0005$. Two-way ANOVA did not show a significant interaction between diet and treatment for Nrf2 protein, but did reveal main effects of diet and LPS treatment. Bonferroni post-hoc analysis comparing Saline ($n = 9$) vs LPS ($n = 8$) and LPS vs LPS + SP0.1% ($n = 14$) groups are shown. *denotes statistical significance (* $p < 0.05$; ** $p < 0.01$). Values are expressed as means $\pm$ SEM.

Figure 7.

Effects of maternal SP0.1% diet on LPS (PND5) induced changes of phosphorylated p38 and acetylated histone3 protein expression in the pup cerebral cortex (PND12) (see Fig. 1A-B for details). Protein expression levels of pp38 were normalized to total p38 (Fig. 7A) and acetylated histone H3 levels were normalized to tubulin (Fig. 7B), the respective densitometric analyses of Western blot are depicted in Fig. 7C and Fig. 7D. Two-way ANOVA showed a statistically significant interaction between diet and LPS treatment for acetylated histone H3, $p = 0.0328$. Two-way ANOVA did not show a significant interaction between diet and treatment for pp38, but did reveal main effects of diet and LPS treatment. Bonferroni post-hoc analysis comparing Saline ($n = 9$) vs LPS ($n = 8$) and LPS vs LPS + SP0.1% ($n = 14$) groups are shown. *denotes statistical significance (* $P < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns-not significant). Values are expressed as means $\pm$ SEM.

Figure 8.

A. Effect of LPS (PND5) on expression of miRNA 146a in cerebral cortex at PND8, PND12, PND35 and PND70. B. Effects of the SP0.1% diet to lactating mothers on LPS (PND5) induced changes of miRNA 146a in pup cerebral cortex (PND12) (see Fig. 1A-B 2.1-2.3 for details). In Fig. 8A values are expressed as fold change over control, $n = 8-10$. *Indicates significant differences ($p < 0.05$) using the Mann–Whitney U test. In Fig. 8B, two-way ANOVA showed a statistically significant interaction between diet and LPS treatment for miRNA146a, $p = 0.0011$. Bonferroni post-hoc analysis comparing Saline ($n = 9$) vs LPS ($n = 8$) and LPS vs LPS+SP0.1% ($n = 14$) groups are shown, *denotes statistical significance (* $p < 0.05$; *** $p < 0.001$; ns-not significant). Values are expressed as means $\pm$ SEM.

Table 1

List of primers used for qRT-PCR

Primer name	Catalog number
Rn_I11b_1_SG QuantiTect Primer Assay	QT00181657
Rn_Tnf_1_SG QuantiTect Primer Assay	QT00178717
Rn_Casp1_1_SG QuantiTect Primer Assay	QT00191814
(Nrf2) Rn_RGD:620360_1_SG QuantiTect Primer Assay	QT00183617
Rn_Keap1_1_SG QuantiTect Primer Assay	QT00189595
Rn_Gclc_1_SG QuantiTect Primer Assay	QT00178878
Rn_Gclm_1_SG QuantiTect Primer Assay	QT00180103
Rn_Hmox1_1_SG QuantiTect Primer Assay	QT00175994
Rn_Ppargc1a_1_SG QuantiTect Primer Assay	QT00189196
Rn_Gusb_1_SG QuantiTect Primer Assay	QT01082053
Rn_Gapd_1_SG QuantiTect Primer Assay	QT00199633
Rn_Hprt1_2_SG QuantiTect Primer Assay	QT00365722
Rn_miR-146_1 miScript Primer Assay	MS00000441
Rn_miR-16_2 miScript Primer Assay	MS00033229