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Detoxication enzyme inducers modify cytokine production in rat mixed glial cells

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Abstract

Pro-inflammatory cytokines, e.g. interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNFα) as well as neurotoxic molecules such as nitric oxide (NO), that are produced and released by activated glial cells, play an important role in inflammation and oxidative stress occurring during Multiple Sclerosis (MS). Reduction of these processes could therefore be of therapeutic interest. Dimethylfumarate (DMF) and sulforaphane (SP) are well known for their detoxicating properties. Furthermore, they have anti-inflammatory effects as shown clinically by the treatment of inflammatory skin diseases. However, their detoxication and anti-inflammatory action on brain-derived cells is unknown. In the present study we have studied, within the same concentration range, the anti-inflammatory and detoxicating effects of DMF and SP on the production and release of mediators of inflammation and detoxication from lipopolysaccharide (LPS) activated primary co-cultures of rat microglial and astroglial cells. DMF and SP attenuated the LPS-induced production and release of TNFα, IL-1β, IL-6 and NO. In addition, DMF and SP increase both mRNA level and activity of NAD(P)H:quinone reductase (NQO-1), a detoxication enzyme, as well as the cellular glutathione content. We conclude that DMF or SP simultaneously can (1) reduce mediators of inflammation and (2) enhance detoxication enzymes in LPS stimulated co-cultures of astroglial and microglial cells. This double-sided effect could potentially be of therapeutic interest.

Keywords: Interleukin-1β; Interleukin-6; Tumor necrosis factor-α; Nitric oxide; Dimethylfumarate; Sulforaphane

1. Introduction

The etiology of chronic inflammatory, neurodegenerative diseases such as Multiple Sclerosis (MS) is not well understood. Neuropathologically, the presence of infiltrated immune cells and activated glial cells is evident (Benveniste, 1997; Heneka and Feinstein, 2001). During inflammation these cells produce a variety of pro-inflammatory mediators including interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factors-α (TNFα) as well as neurotoxic molecules such as nitric oxide (NO). All of these are known to contribute directly or indirectly to cellular dysfunction and/or cell death associated with neurodegenerative processes (Hantraye et al., 1996;

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Hooper et al., 1997; Oshima et al., 1996; Simonian and Coyle, 1996; Takahashi et al., 1997). In MS for example, cytokines and reactive oxygen species (ROS, including NO) are present in perivascular T-cells, macrophages and in surrounding activated glial cells. It is shown that their presence leads to demyelination, functional motor disturbances and finally axonal loss (Penkowa and Hildago, 2003; Bar-Or et al., 1999; Brosnan et al., 1995; Cannella and Raine, 1995).

Neuro-inflammation and oxidative stress, as detected in MS, leads to alterations in the activity/functionality of resident brain cells. In a chronic situation, it has a progressive effect on the course of the disease, emphasizing the importance of reducing the level of inflammation and reactive oxygen species within the brain. Several naturally occurring and synthetic compounds, including dimethylfumarate (DMF) and sulforaphane (SP), are known to present detoxicating properties in mammalian cells. These compounds do not scavenge free radicals, but elevate the production of relevant detoxication enzymes including NAD(P)H:quinone reductase (NQO-1) and/or stimulate glutathione synthesis (Basten et al., 2002; Dringen et al., 1998; Gao et al., 2001; Khanna et al., 1998; McMahon et al., 2001; Nelson et al., 1999; Prochaska and Fernandes, 1993; Spencer et al., 1990).

Interestingly, DMF and SP have also been shown to affect inflammatory processes. DMF has been used for the systemic treatment of psoriasis and reduces itching and scaling and prevents extension of the disease (Nieboer et al., 1989). Mechanistically, DMF reduces cytokine expression in lymphocytes (de Jong et al., 1996; Ockenfels et al., 1998), has an anti-proliferative effect on keratinocytes (Thio et al., 1994) and, inhibits monocyte differentiation into dendritic cells (Zhu and Mrowietz, 2001). SP down-regulates bacterial lipopolysaccharide (LPS)-mediated production of several inflammatory proteins (e.g. iNOS, COX-2 and TNFα) in cultured macrophages (Heiss et al., 2001). Recently, Wu and collaborators have shown decreases in $NF_{\kappa}B$ nuclear translocation and infiltration of activated macrophages in kidneys of spontaneous hypertensive rats fed with dried broccoli sprouts containing glucoraphanin, metabolized into SP (Wu et al., 2004).

The compounds DMF and SP may represent promising therapeutic targets to enhance detoxication and reduce inflammation, if both actions occur within the same concentration range of the compound used. In the present study, we question whether DMF and SP show detoxication and anti-inflammatory activities in LPS-activated brain-derived glial cells. Until now, little is known of effects of DMF and SP on brain-derived cells. To address this question, rat mixed microglia and astrocytes were treated with LPS to stimulate production of pro-inflammatory mediators (e.g. IL-1β, TNFα, IL-6) and reactive oxygen species (e.g. NO). Subsequently, the anti-inflammatory effects of DMF and SP on IL-1β, TNFα, IL-6 and NO production by the glial cells were studied. In addition, the detoxicating effects of DMF and SP were studied by measuring glutathione (GSx) levels and NQO-1 activity in the cells.

2. Materials and methods

2.1. Mixed glial cell culture

Primary co-cultures of astroglial and microglial cells were prepared from newborn (2 day-old) Wistar rats (Harlan CPB, Zeist, The Netherlands) (Vincent et al., 1996). Cerebral cortices were cleared from adhering meninges and blood vessels, and dissociated using 0.25% trypsin (Sigma Chemical Co., St Louis, MO) in phosphate-buffered saline (PBS). Cells (0.5×10^6/well) were plated in poly-L-lysine (15 μg/ml; Sigma)-coated 12 wells plates (Nunclon, Hamstrop, Denmark) and incubated at 37 °C in humidified air containing 5% CO_2. The culture medium consisted of Dulbecco's modified Eagles medium (DMEM)-F10 (Gibco, Life Technologies, Breda, The Netherlands), supplemented with 10% v/v heat-inactivated fetal calf serum (FCS) (Gibco), 2 mM L-glutamine (Sigma), 37.5 U/ml streptomycin (Sigma) and 100 U/ml penicillin (Sigma). The medium was changed 1 day after seeding. Cultures consisted mainly of microglial and astroglial cells with less than 0.01% oligodendrocytes. Experiments were carried out after 7 days of culture, when microglia showed short ramifications and cultures were confluent.

2.2. Treatment of glial cells

In a first series of experiments, co-cultures were incubated for 1, 2, 4, 6, 8, 16, 24, 48 or 72 h with LPS (100 ng/ml; *Escherichia coli* serotype 055-B5, Westphal, Difco, Detroit, MI) or media alone to determine the time-dependent cytokine production by activated glial cells. Culture supernatants were collected, divided into aliquots and stored at −20 °C until assayed. The cells were lysed with a RNA extraction buffer (Promega Corporation, USA) and stored at −80 °C. In subsequent series of experiments, astroglial–microglial cell co-cultures were incubated for 4 and 24 h with LPS (100 ng/ml). Incubation with LPS was carried out in the absence (vehicle: 0.0006% DMSO or 50 μM HCL) or presence of DMF (5, 15 or 30 μM in 0.0006% DMSO) or SP (1, 5 or 15 μM in 50 μM HCL). All conditions were tested in triplo and the experiments were repeated at least twice. Treatment of the cells did neither result in reduced cell viability nor in altered cellular protein levels within the timeframe of the experiments (data not shown). After incubation, culture supernatants were collected, divided into aliquots and stored at −20 °C. Cells were lysed with a RNA extraction buffer (Promega Corporation) and stored at −80 °C or lysed with Tris/EDTA and sonificated for NQO-1 activity and stored at −20 °C. For GSx measurements, cells were lysed with 2.5% salicilyc acid (SSA) and stored at −20 °C.

Table 1
Rat primers and accession numbers of the rat DNA sequences used to design the primers

Name	Primer FW	Primer Rev	Accession number
GAPDH	5′-GAACATCATCCCTGCATCCA-3′	5′-CCAGTGAGCTTCCCGTTCA-3′	NM_017008
IL-1β	5′-AAAGAAGAAGATGGAAAAGCGGTT-3′	5′-GGGAACTGTGCAGACTCAAACTC-3′	NM_031512
IL-6	5′-CCCCAACTTCCAATGCTCTC-3′	5′-AGATGAGTTGGATGGTCTTGGTC-3′	NM_012589
iNOS	5′-AACTTGAGTGAGGAGCAGGTTGA-3′	5′-CGCACCGAAGATATCCTCATGA-3′	NM_012611
NQO-1	5′-AACGTCATTCTCTGGCCAATTC-3′	5′-GCCAATGCTGTACACCAGTTGA-3′	NM_017000
TNFα	5′-CCACACCGTCAGCCGATT-3′	5′-TCCTTAGGGCAAGGGCTCTT-3′	AJ002278

2.3. Nitrite assay

The nitrite concentration in the culture supernatant is used as a measure of NO production. Nitrite was measured by a colorimetric assay based on the Griess reaction, as described by Ding et al. (Ding et al., 1988). Briefly, 100 μl supernatant was mixed with 100 μl Griess reagent, consisting of 1% sulfanilamide (Jansen chimica, Geel, Belgium), 0.1% naphtylethylene diamine dihydrochloride (Merck, Darmstadt, Germany), and 2.5% H_3PO_4 in water, and incubated at room temperature (RT) for 10 min. The absorbance was measured in a microtiter plate reader at 550 nm (ICN Flow, MS2 Reader, ICN Biomedicals, Zoetermeer, The Netherlands). Sodium nitrite (Merck) dissolved in culture medium was used as the standard. Detection limit of the assay was 1 μM nitrite.

2.4. Rat IL-6 ELISA

IL-6 concentrations were measured in culture supernatant using an enzyme-linked immunosorbent assay (ELISA) specific for rat IL-6 (Rees et al., 1999a). Immunoaffinity-purified sheep anti-rat IL-6 antibodies were used as coating and detecting antibodies (S206/B1:2 mg/ml and 1:1000 dilution, respectively; NIBSC Potter Bar, UK). Recombinant rat IL-6 (NIBSC) diluted in culture medium/HPE (CLB, Amsterdam, The Netherlands) (50% v/v) was used as the standard. Detection limit of the assay was 8 pg IL-6/ml.

2.5. Rat IL-1β ELISA

The concentration of IL-1β was measured using a sandwich ELISA specific for rat IL-1β (Safieh-Garabedian et al., 1995). Purified polyclonal sheep anti-rat IL-1β antibodies were used as coating and detecting antibodies (S1002(110700): 1 μg/ml and 1:1000 dilution, respectively; NIBSC). Standard curves, included in duplicate on each plate, were made with rat recombinant IL-1β (NIBSC) diluted in culture medium/HPE (CLB) (50% v/v) to measure IL-1β in the supernatant. Detection limit of the assay was 10 pg IL-1β/ml.

2.6. Rat TNFα ELISA

The concentration of TNFα was measured using a sandwich ELISA specific for rat TNFα (Rees et al., 1999b). Purified polyclonal sheep anti-rat TNFα antibodies were used as coating and detecting antibodies (S54(050900): 1 μg/ml and 1:500 dilution, respectively; NIBSC). Standard curves, included in duplicate on each plate, were made with rat recombinant TNFα (NIBSC) diluted in culture medium/HPE (CLB) (50% v/v). Detection limit of the assay was 10 pg TNFα/ml.

2.7. NQO-1 enzyme activity measurement

Cells were washed with PBS and lysed in ice cold 25 mM Tris–HCL and 1 mM EDTA (pH=7.4). After 5 s of

Table 2
IL-1β, IL-6, TNFα and nitrite levels produced by mixed astroglial–microglial cells

Protein	1 h	2 h	4 h	6 h	8 h	24 h	48 h	72 h
IL-1β (pg/ml)								
Control	<dl	<dl	<dl	<dl	<dl	<dl	<dl	<dl
LPS	<dl	3.53±0.55	12.77±1.44	30.80±3.97	36.57±9.13	75.83±5.91	83.33±3.79	123.13±10.61
IL-6 (pg/ml)								
Control	<dl	<dl	<dl	<dl	<dl	<dl	<dl	<dl
LPS	<dl	<dl	392.70±18.23	870.93±92.55	1019.83±88.78	3711.67±88.50	5164.60±245.22	5775.50±451.42
TNFα (pg/ml)								
Control	<dl	<dl	<dl	<dl	<dl	<dl	<dl	<dl
LPS	139±62.13	1167±96.42	3110±97.54	3612±152.26	3716.1±292.10	4274.267±165.39	1918.6±97.92	1275.6±101.33
Nitrite (μM)								
Control	<dl	<dl	<dl	<dl	<dl	<dl	<dl	<dl
LPS	<dl	<dl	<dl	<dl	<dl	6.97±0.51	24.10±1.23	35.17±1.00

<dl=below detection limit.

sonification, cytosolic supernatants were collected, aliquoted and stored at −20 °C prior to use.

The enzymatic activity of NQO-1 was determined spectrophotometrically by measuring the NADPH-dependent, menadiol-mediated reduction of 3-(4,5-dimethylthiazol-2yl)-2,5-diohenyltetrazolium bromide (MTT) as described before (Prochaska and Santamaria, 1988) with minor modifications (van Muiswinkel et al., 2000).

2.8. Cellular localization of NQO-1

Mixed glial cells were cultured in a 12 wells plate and treated for 24 h with LPS (100 ng/ml), LPS and DMF (30 μM) or LPS and sulforaphane (15 μM). After treatment, the cells were washed with PBS and fixed for 20 min with 4% paraformaldehyde. The cells were rinsed with TBS and subsequently incubated for 1 h at room temperature with a rabbit anti-rat NQO-1 antibody (MS-14; Dr. M. Schultzberg, Karolinska Institute, Stockholm, Sweden), diluted 1 : 10,000 in TBS containing 0.1% Tween-20, followed by an incubation with biotinylated goat anti-rabbit IgGs (1 : 100; Jackson Immunoresearch Laboratories, USA) and finally an incubation with ABC (1 : 400; Vector Laboratories, USA). After a final wash in Tris/HCl, a nickel-enhanced DAB reaction was performed using H_2O_2 as a substrate to identify NQO-1 positive cells.

A double-labeling immunocytochemical procedure was performed to determine which glial cell type expresses NQO-1. Therefore, the cells were incubated with a mixture of either rabbit anti-rat NQO-1 (1 : 10,000) and biotinylated mouse anti-rat ED-1 (1 : 250; gift from Prof. Dr. C.D. Dijkstra, Department of Molecular Cell Biology, VUmc, Amsterdam) or rabbit anti-rat NQO-1 (1 : 10,000) and goat anti-rat GFAP (1 : 100; Santa Cruz). After washing, the cells were incubated with either Texas Red labelled goat anti-rabbit IgGs (1 : 100; Jackson Immunoresearch Laboratories) and Alexa fluor 488 labelled streptavidin (1 : 100; Molecular Probes, or biotinylated donkey anti-rabbit IgGs (1 : 100; Jackson Immunoresearch Laboratories) and FITC labelled donkey anti-goat IgGs (1 : 25; Nordic, Denmark). Finally, the cells double-labelled for NQO-1 and GFAP were incubated with Texas Red labelled avidin (1 : 100; Vector Laboratories).

2.9. Glutathione level measurement

To measure the total intracellular glutathione (GSx = 1 GSH + 2 GSSG) content in cell lysates, we used a spectrophotometrical assay as described previously (Drukarch et al., 1996). The amount of GSx in a cell lysate was determined according to a standard curve of 0, 1, 2, 3, 4 and 5 μM GSx in 2.5% SSA, and calculated as μmol/mg protein.

2.10. RNA extraction

Cells were homogenized in 175 μl of SV RNA Lysis Buffer (SV Total RNA Isolation System, Promega Corporation, USA). Total RNA was extracted according to the manufacturer's protocol. The RNA samples were eluted in sterile water and concentrations were measured at A_{260} with a spectrophotometer (SPECTRA$_{max}$ 250, Molecular Devices, USA). The purity of RNA was valued on by a ratio A_{260}/A_{280} of 1.9–2.1.

2.11. Reverse transcription

One microgram of total RNA was reverse transcribed using the Avian Myeloblastosis Virus (AMV) Reverse Transcriptase (Reverse Transcription System, Promega Corporation). The absence of contaminating genomic

Table 3
Relative mRNA expression of IL-1β, IL-6, TNFα, iNOS and NQO-1 measured in mixed astroglial–microglial cells

mRNA	1 h	2 h	4 h	6 h	8 h	24 h	48 h	72 h
IL-1β (Relative expression to GAPDH, in percent)								
Control	0.46±0.03	0.64±0.002	0.67±0.03	0.26±0.07	0.30±0.13	0.07±0.004	0.08±0.01	0.16±0.02
LPS	77.23±1.56	130.15±6.32	233.55±3.76	163.62±2.62	151.4±5.26	151.07±5.39	118.33±5.22	119.12±4.88
IL-6 (Relative expression to GAPDH, in percent)								
Control	0.21±0.0001	0.22±0.02	0.11±0.003	0.11±0.01	0.09±0.01	0.04±0.01	0.01±0.001	0.03±0.001
LPS	1.35±0.002	16.07±1.03	67.7±3.43	21.21±0.93	19.85±1.4	19.19±0.67	13.72±1.00	11.14±0.6
TNFα (Relative expression to GAPDH, in percent)								
Control	0.62±0.05	0.65±0.03	0.43±0.03	0.30±0.03	0.33±0.04	0.14±0.01	0.14±0.01	0.12±0.01
LPS	51.29±2.49	64.96±3.50	39.89±2.14	9.09±0.46	9.64±0.29	3.05±0.14	1.49±0.05	0.92±0.07
iNOS (Relative expression to GAPDH, in percent)								
Control	0.0075±0.0008	0.0123±0.004	0.0166±0.0009	0.0093±0.0006	0.0040±0.001	0.0002±0.0001	0.0002±0.0002	0.0005±0.0001
LPS	0.04±0.01	0.76±0.07	10.95±0.85	8.41±0.32	3.77±0.04	12.05±0.12	4.75±0.10	3.89±0.51
NQO-1 (Relative expression to GAPDH, in percent)								
Control	1.55±0.05	1.69±0.04	1.61±0.07	1.81±0.04	1.68±0.04	1.80±0.16	1.10±0.09	1.95±0.27
LPS	1.61±0.01	1.70±0.01	1.45±0.06	1.29±0.04	1.43±0.06	3.66±0.09	4.57±0.15	4.56±0.44

DNA and the efficiency of the RT reactions were controlled by PCR on RNA and cDNA using GAPDH Forward and Reverse primers (Table 1).

2.12. Quantitative PCR

The cDNA synthesized was assayed using quantitative real time PCR (SYBR Green PCR, ABI 7700, PE Applied Biosystem, California, USA). With this method, direct detection of PCR products is monitored by measuring the increase in fluorescence caused by the binding of SYBR Green to double stranded DNA. The resulting relative increase in reporter fluorescent dye emission was monitored in real time during PCR amplification using the Sequence Detection System (ABI PRISM 7700 Sequence Detection system and software, PE Applied Biosystems).

Primers (Table 1) were designed from rat corresponding sequences using Primer Express Software (PE Applied Biosystems).

Quantitative PCR was carried out in a final volume of 20 µl with 6.5 ng of cDNA, 3 mM $MgCl_2$, 0.2 mM dATP, dCTP and dGTP, 0.4 mM dUTP, 0.75 mM of each primer, 0.3 U of AmpliTaq Gold DNA and 0.12 U of Amperase uracil-*N*-glycosylase (all reagents from PE Applied Biosystem). PCR conditions were: 50 °C for 2 min, 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min.

Threshold cycle (C_t) values provide an index of the mRNA level. The level of GAPDH mRNA was used as an internal standard to control the amplification variations due to differences in the starting mRNA concentrations. The relative expression level of cytokine mRNA for each tissue was computed from the C_t values obtained for the gene of interest and GAPDH using the following formula:

Relative mRNA expression of gene of interest (in percent compared to the GAPDH)

$$= 2^{-(C_t \text{ interest gene} - C_t \text{ GAPDH})}$$

2.13. Statistical analysis

Using the NCSS 2000 statistical program (NCSS, Kaysville, Utah, US), two-way ANOVA was performed

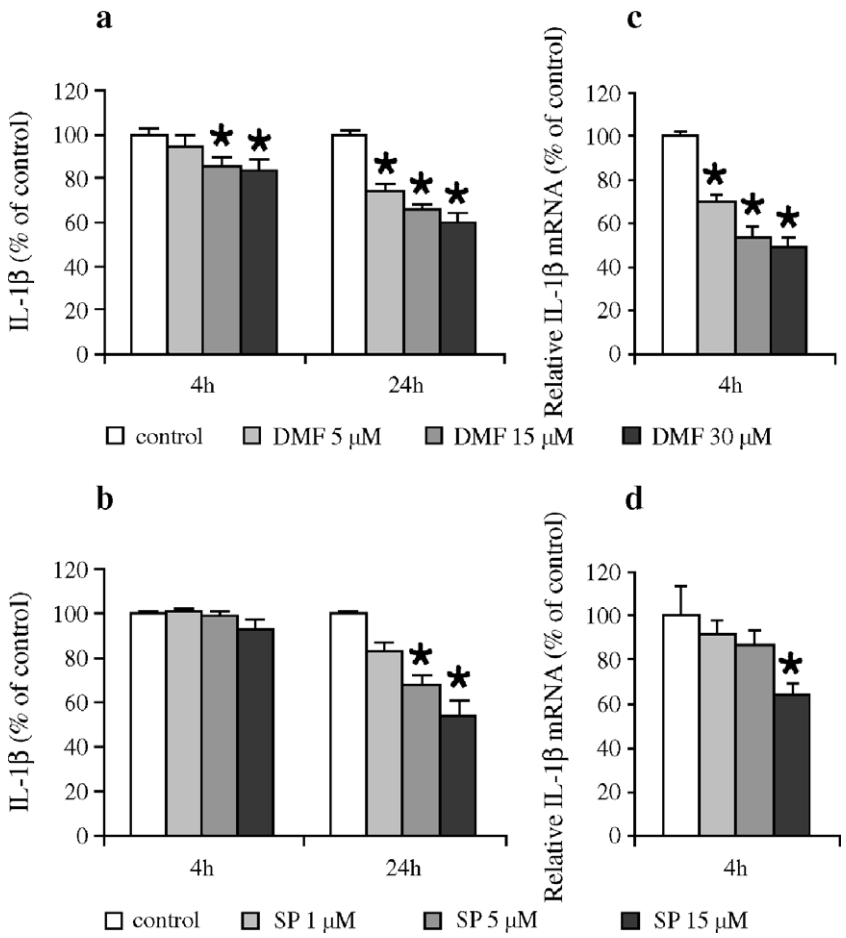

Fig. 1. Effects of DMF and SP on lipopolysaccharide (LPS)-induced production of IL-1β in mixed glial cells. Cells were treated with LPS (100 ng/ml) for 4 and 24 h. IL-1β protein was measured in the media after 4 and 24 h and IL-1β mRNA was measured after 4 h. a, effect of DMF on IL-1β protein level, b, effect of SP on IL-1β protein level, c, effect of DMF on IL-1β mRNA level, d, effect of SP on IL-1β mRNA level. Data are expressed as percentage of levels measured in cultures co-incubated with LPS and solvent (control), and represent mean±S.E.M. of 2–3 independent experiments (n=6–12). *P<0.01 vs. control.

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