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Accelerated degradation of retinoic acid by activated microglia

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ABSTRACT

In the brain, retinoic acid (RA) concentrations are under tight spatio-temporal control. Here, we show that challenge of primary mouse microglia with lipopolysaccharide (LPS) results in increased release of nitric oxide (NO) and tumor necrosis factor-α (TNF-α). Co-administration of RA attenuated microglial activation. Similarly, pretreatment with RA-metabolism inhibitor liarozole potently reduced NO and TNF-α release. Conversely, activated microglia showed increased protein expression of RA-degrading cytochromes CYP26A1, CYP26B1, CYP3A4 and CYP2C. Correspondingly, RA catabolism by activated microglia was significantly increased. Our results indicate that RA reduces microglial activation, but also, conversely, that the activation state of microglia influences RA metabolism.

1. Introduction

Microglia serve as the primary resident immune cells of the brain. Microglial activation is a crucial biological process that occurs after stimulation with e.g. bacterial surface antigens or certain cytokines (Ricciardi-Castagnoli et al., 1990; Gehrmann et al., 1995a, 1995b; Lynch, 2009). Microglial activation is characterized by morphological and functional changes. Activated microglia secrete proinflammatory mediators such as TNF-α and interleukin 1β (Hetier et al., 1990; Davies et al., 1999). Furthermore, activated microglia produce reactive oxygen species (ROS) and nitric oxide (NO) (Graeber, 2010; Graeber and Streit, 2010).

Microglial activation is a hallmark of a variety of neurodegenerative diseases including stroke, multiple sclerosis, Parkinson's disease and Alzheimer's dementia. Indeed, microglial activation is discussed as a crucial contributing factor in the etiopathogenesis of these diseases (Langston et al., 1999; Hanisch, 2002; Graeber, 2010; Graeber and Streit, 2010). Importantly, modulation of the state of microglial activation may confer neuroprotection under certain conditions (Liu et al., 2011; Ossola et al., 2011; Zhu et al., 2011). Finally, a number of studies have described effects of microglia-preconditioned media on neuronal differentiation, viability and function (e.g. Hetier et al., 1990; Munch et al., 2003; Schlichter et al., 2010).

Retinoic acid (RA), acting through nuclear retinoic acid receptors RAR and RXR, is a key regulator of neuronal development, differentiation and survival (Maden, 2007). Moreover, RA exerts potent anti-inflammatory effects under conditions of neuroinflammation (Dheen et al., 2005; Xu et al., 2005, 2006; Kim et al., 2008; van Neerven et al., 2010; Matsushita et al., 2011). RA levels in the brain are under tight spatio-temporal control (White et al., 1996; Abu-Abed et al., 2001; Stoilov et al., 2001; Reijntjes et al., 2007; Xi and Yang, 2008; Thatcher and Isoherranen, 2009; Olson and Mello, 2010). Surprisingly, the mechanisms of local RA regulation in neural tissue have so far gained rather little attention either in the context of physiological neuronal homeostasis or brain pathology. Although a direct effect of activated microglia on RA concentrations could contribute to altered neuronal responses upon exposure to culture media conditioned by activated microglia, reports on microglial RA metabolism are still lacking. Similarly, the effects of the pharmacological inhibition of endogenous microglial RA catabolism on microglial function have not yet been investigated. Here, we show that LPS-activated microglia exhibit increased degradation of RA in culture medium along with increased protein expression of cytochromes CYP26A1, CYP26B1, CYP3A4 and CYP2C, which are known to participate in RA degradation. Furthermore, pharmacological inhibition of intracellular RA degradation with liarozole, a specific RA metabolism blocking agent, exerted potent anti-inflammatory effects on activated microglia.

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2. Methods

2.1. Primary microglial cultures

Cultures of primary mouse microglia were prepared from newborn C57BL/6N mice as described in detail recently (Regen et al., 2011). Microglial cells were harvested by gentle shake off and placed in either 6- or 12-well plates at an initial density of 10^6 or 3×10^5 cells per well, respectively. Cells remained in culture for an additional 24 h before use. The purity of cultures exceeded 98% as verified by regular flow cytometry analyses with CD11b and CD45 staining (rat anti-mouse CD11b and rat anti-mouse CD45; both from BD Pharmingen, Heidelberg, Germany). All experiments were performed in DMEM (Invitrogen/Gibco, Karlsruhe, Germany) containing 10% fetal calf serum (FCS; Biochrom, Berlin, Germany). LPS (*Escherichia coli* 055:B5) and all-trans RA (both from Sigma-Aldrich, St. Louis, U.S.A) were applied at concentrations of 1 μg/ml or 100 ng/ml and 10 μM, respectively. Liarozole (Tocris, Bristol, U.K.) was applied at a final concentration of 50 μM. Due to the photosensitivity of retinoids, all experiments were performed under dim, indirect illumination.

2.2. Measurement of nitric oxide release

Nitric oxide (NO) production was quantified as nitrite accumulation using the Griess reagent for nitrite (Sigma-Aldrich). 100 μl of cell culture supernatant was incubated with 100 μl Griess reagent. Absorption was measured at 550 nm with a microplate spectrophotometer (TriStar, Berthold Technologies, Bad Wildbad, Germany). The concentration of nitrite in samples was calculated using a standard curve. Griess reagents showed no cross-reactivity with liarozole as also confirmed by a standard curve.

2.3. Analysis of TNF-α

The production of TNF-α was quantified using a mouse TNF-α specific enzyme-linked immunosorbent assay (eBioscience, Frankfurt, Germany) according to the manufacturer's instructions.

2.4. MTT assay

Cell viability was assayed by measuring the intracellular reduction of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) to formazan. The MTT labeling agent (Sigma-Aldrich, Germany) was added to the cells at a final concentration of 0.5 mg/ml. The formazan product was solubilized in 10% SDS in 0.01 M HCl and measured at 550 nm with a plate reader.

2.5. Retinoic acid (RA) measurements

Cell culture supernatants were collected under dim, indirect light and immediately frozen for storage at −80 °C until further analysis within 4 weeks. For retinoid extraction, a rapid, single-step extraction and analysis procedure was developed. Retinoic acid was quantified as described in detail previously (Hellmann-Regen et al., 2012). In brief, 4 vol. of ice-cold methanol (MeOH) was added to 1 vol. of cell culture supernatant and vortexed. Samples were subsequently kept at −20 °C for achieving thorough protein precipitation. After centrifugation at 4 °C and 14,000 rpm for 10 min, the resulting supernatant was subjected to reversed-phase HPLC analysis (Agilent 1100 model liquid chromatography system equipped with a 1290 Infinity diode array detector; Agilent Technologies, Böblingen, Germany).

2.6. Immunoblotting

Cells were pooled from three independent samples and lysed with ice-cold mammalian protein extraction reagent (Pierce Biotechnology, Rockford, Ill., U.S.A.). Cellular debris was precipitated by centrifugation at 20,000 *g* and 4 °C for 25 min. Protein concentration was determined using BCA assay (Pierce Biotechnology). Equal amounts of protein were loaded on sodium dodecyl sulfate polyacrylamide gels (10–20%) and blotted onto PVDF membranes (Millipore, Schwalbach, Germany). Blots were probed with specific antibodies directed against cytochrome P450 3A4 (1:500; Abcam, Cambridge, U.K.), CYP26A1 (1:1000, Life Span Biosciences, Seattle, U.S.A.), CYP26B1 (1:1000, Life Span Biosciences), CYP P450 2C family (1:1000; Abcam) and co-probed using a pre-labeled anti-β-actin antibody (HRP-labeled, 1:60,000; Sigma).

2.7. Quantitative real-time reverse-transcription polymerase chain reaction

Cells were homogenized and total RNA was extracted using TRIzol® reagent (Invitrogen). For polymerase chain reaction (PCR) amplification, we used gene-specific primers (Table 1) in a Light Cycler® system with SYBR Green I fluorescence monitoring for template quantification (Roche Diagnostics). Crossing points of amplified products were determined using the Second Derivative Maximum Method. Target mRNA was quantified relative to tripeptidyl peptidase (Tpp) 2 (Nishida et al., 2006; Gertz et al., 2012). Specificity of PCR was checked using melting curve analysis and electrophoresis in a 1.5% agarose gel. Primer sequences are given in Table 1.

2.8. Statistical analysis

Values are given as means ± standard deviations from at least three independent experiments. HPLC measurements were performed in duplicate for each sample. All numerical analyses were performed using the statistical software PASW Statistics 18, Release 18.0.0.0 (IBM Corporation, Somers, N.Y., U.S.A.). Differences between group means were analyzed by one-way ANOVA followed by Tukey's post-test or two-way ANOVA followed by Bonferroni multiple comparison post-test where appropriate. $p<0.05$ was considered statistically significant.

3. Results

Primary mouse microglia were exposed to LPS. LPS treatment resulted in microglial activation as evidenced by significantly increased NO and TNF-α release. In line with an earlier study in rats (Dheen et al., 2005), co-treatment with RA attenuated the activating effect of LPS on microglia (Fig. 1A,B). Liarozole represents a novel class of molecule known as retinoic acid metabolism blocking agent (RAMBA). It potently inhibits the intracellular cytochrome P450-dependent catabolism of RA (Van Wauwe et al., 1990, 1992; Kang et al., 1996). Liarozole exerted strong retinoid-mimetic effects on NO and TNF-α release of LPS-activated microglia. Importantly, liarozole proved highly effective in spite of the fact that no exogenous RA had been added to the cultures. We therefore measured RA concentrations in fetal calf serum, the

Table 1
Primer sequences used in this study.

Target gene	Direction	Sequence (5′→3′)
Rara	Forward	gct tct tcc gac gaa gca tcc ag
	Reverse	gat cgt ttc gca ccg act cct tg
Rarb	Forward	gct tcg ttt gcc agg aca agt c
	Reverse	cat agc tct ctg tgc att cct gc
Rarg	Forward	caa gtc ttc tgg cta cca cta tg
	Reverse	gtt cct tac agc ttc ctt gga c
Rxra	Forward	cac caa aca ttt cct gcc gct c
	Reverse	ctc agg gtg ctg ata gga gag t
Rxrb	Forward	cga agc tca ggc aag cac tat g
	Reverse	cat ctc cat ccc cgt ctt tgt c
Rxrg	Forward	ctc tgg tga aac aca tct gtg c
	Reverse	ctg tct ttg gtt cca cag caa g
tpp-2	Forward	ctt cta tcc aaa ggc tct caa gg
	Reverse	ctc tcc agg tct cac cat cat g