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Short communication

Aging sensitizes rapidly isolated hippocampal microglia to LPS ex vivo

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ABSTRACT

The present study tested whether aging sensitizes hippocampal microglia to a pro-inflammatory challenge ex vivo. Hippocampal microglia from 3 and 24 mo old male F344×BN F1 rats were exposed to LPS (0, 0.1, 1, 10 and 100 ng/ml) ex vivo. 2 h post-LPS challenge, gene expression of microglial activation markers and cytokines were assessed. 24 mo old animals exhibited a potentiated pro-inflammatory cytokine (IL-1β and IL-6) response to LPS and increased levels of CD11b, Iba-1 and MHCII irrespective of LPS treatment. The present results demonstrate that aging sensitizes hippocampal microglia to pro-inflammatory challenges.

1. Introduction

In normal aging, the CNS exhibits a sensitized neuroinflammatory response to peripheral as well as central administration of proinflammatory agents (Abraham et al., 2008; Abraham and Johnson, 2009; Barrientos et al., 2009; Barrientos et al., 2006; Chen et al., 2008; Godbout et al., 2005; Henry et al., 2008; Henry et al., 2009; Huang et al., 2008). Microglia are critical to the mediation of neuroinflammation (Kreutzberg, 1996) and it has been suggested that aging "primes" microglia so that they produce exaggerated levels of inflammatory mediators, such as IL-1β, when stimulated (Dilger and Johnson, 2008), although direct evidence is lacking. We (Barrientos et al., 2009) and others (Abraham and Johnson, 2009) have focused on the age-related vulnerability of the hippocampus as this CNS structure appears particularly susceptible to exaggerated pro-inflammatory responses in the face of a peripheral immune challenge. Thus, for example, hippocampal IL-1β increases following peripheral infection are potentiated in aging subjects (Barrientos et al., 2009), but there is no direct evidence that the hippocampal microglia are actually sensitized. In the present study, we determined whether hippocampal microglia are indeed sensitized by aging by rapidly separating microglia from hippocampus using a procedure that we have previously developed (Frank et al., 2006b) and stimulating them ex vivo.

2. Materials and methods

2.1. Subjects

3 and 24 mo old male F344×BN F1 rats ($N=4$/group) were utilized. Animals were obtained from the National Institute on Aging (Bethesda, MD). The animal colony was maintained at 22 °C on a 12-h light/dark cycle (lights on at 07:00 h). All rats were allowed free access to food and water and were given 1 week to acclimate to colony conditions before experimentation began. All experiments were conducted in accordance with protocols approved by the University of Colorado Animal Care and Use Committee.

2.2. Tissue collection

Animals were given a lethal dose of sodium pentobarbital and transcardially perfused with ice-cold saline (0.9%) for 3 min to remove peripheral immune leukocytes from the CNS vasculature. Brain was rapidly extracted and placed on ice and hippocampus dissected for microglia cell isolation.

2.3. Ex vivo immune stimulation of hippocampal microglia with LPS

Hippocampal microglia were isolated using a Percoll density gradient as previously described (Frank et al., 2006b). In the present experiments, immunophenotype and purity of microglia were assessed using real time RT-PCR. Microglia were suspended in DMEM + 10% FBS and microglia concentration determined by trypan

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blue exclusion. Cell number did not significantly differ between 3 and 24 mo animals. Microglia concentration was adjusted to a density of 5×10^3 cells/100 µl and 100 µl added to individual wells of a 96-well v-bottom plate. LPS was utilized to challenge microglia ex vivo as we have previously determined the optimal in vitro conditions under which LPS stimulates a microglia pro-inflammatory cytokine response (Frank et al., 2006b). Cells were incubated with LPS (0.1, 1, 10, and 100 ng/ml) or media alone for 2 h at 37 °C, 5% CO_2. The plate was centrifuged at 1000×*g* for 10 min at 4 °C to pellet cells and cells washed 1× in ice-cold PBS and centrifuged at 1000×*g* for 10 min at 4 °C. Cell lysis/homogenization, Dnase treatment, and cDNA synthesis were performed using the SuperScript III CellsDirect cDNA Synthesis System (Invitrogen, Carlsbad, CA).

2.4. Real time RT-PCR measurement of gene expression

A detailed description of the PCR amplification protocol has been published previously (Frank et al., 2006b). cDNA sequences were obtained from Genbank at the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov). Primer sequences were designed using the Qiagen Oligo Analysis & Plotting Tool (oligos.qiagen.com/oligos/toolkit.php?) and tested for sequence specificity using the Basic Local Alignment Search Tool at NCBI (Altschul et al., 1997). Primers were obtained from Invitrogen. Primer specificity was verified by melt curve analysis. All primers were designed to exclude amplification of genomic DNA. Primer sequences are as follows: β-Actin, F-TTCCTTCCTGGGTATGGAAT, R-GAGGAGCAATGATCTTGATC; CD11b, F-CTGGTACATCGAGACTTCTC, R-TTGGTCTCTGTCTGAGCCTT; CD163, F-GTAGTAGTCATTCAACCCTCAC, R-CGGCTTACAGTTTCCTCAAG; Glial Fibrillary Acid Protein (GFAP), F-AGATCCGAGAAACCAGCCTG, R-CCTTAATGACCTCGCCATCC; Ionized Calcium Binding Adapter Protein (Iba-1), F-GGCAATGGAGATATCGATAT, R-AGAATCATTCTCAAGATGGC; IL-1β, F-CCTTGTGCAAGTGTCTGAAG, R-GGGCTTGGAAGCAATCCTTA; IL-6, F-AGAAAAGAGTTGTGCAATGGCA, R-GGCAAATTTCCTGGTTATATCC; IL-10, F-GGACTTTAAGGGTTACTTGGG, R-AGAAATCGATGACAGCGTCG; Major Histocompatibility Complex (MHC)II, F-AGCACTGGGAGTTTGAAGAG, R-AAGCCATCACCTCCTGGTAT.

PCR amplification of cDNA was performed using the Quantitect SYBR Green PCR Kit (Qiagen, Valencia, CA). Formation of PCR product was monitored in real time using the MyiQ Single-Color Real-Time PCR Detection System (BioRad, Hercules, CA). Relative gene expression was determined by taking the expression ratio of the gene of interest to β-Actin.

2.5. Statistical analysis and data presentation

All data are presented as mean + SEM. Statistical analyses consisted of ANOVA followed by *t* tests with a Bonferroni correction. Threshold for statistical significance was set at $\alpha = .05$.

3. Results

3.1. PCR phenotyping of microglia

Consistent with our prior work, analysis of cDNA from hippocampal microglia showed robust expression of the microglia markers CD11b, Iba-1 and MHCII, whereas the perivascular/meningeal macrophage marker CD163 failed to amplify by 40 cycles of PCR. Likewise, the astrocyte marker GFAP failed to amplify by 40 cycles of PCR in all samples indicating that the microglia isolation procedure yielded a highly pure microglia population devoid of other CNS macrophages as well as astrocytes. Fig. 1 is a representative amplification plot of each gene through 40 cycles of PCR.

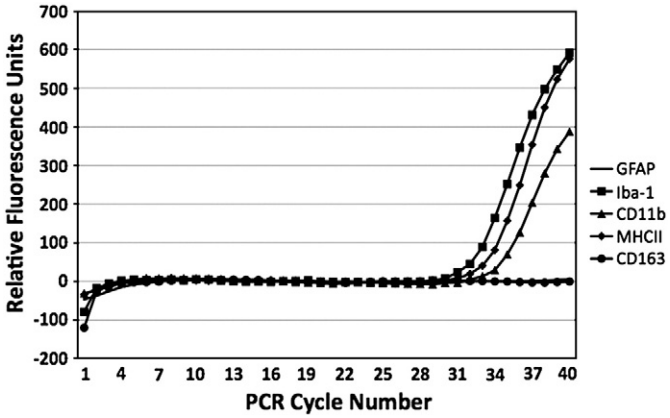

Fig. 1. PCR phenotyping of microglia activation. Hippocampal microglia were isolated and gene expression of several microglia antigens (Iba-1, MHCII, CD11b), perivascular macrophage antigens (CD163) and astrocyte antigens (GFAP) were assayed. Through 40 cycles of PCR, only Iba-1, MHCII, and CD11b amplified, whereas CD163 and GFAP failed to amplify.

3.2. Microglia from 24 mo animals exhibit a potentiated pro-inflammatory cytokine response to LPS

Hippocampal microglia from 3 and 24 mo old animals were exposed to LPS ex vivo to directly test whether aging sensitizes microglia to pro-inflammatory stimuli. Microglia from 24 mo animals exhibited a potentiated IL-1β ($F = 176.9$, 4, 30, $p < .0001$) and IL-6 ($F = 139.0$, 4, 30, $p < .0001$) cytokine response to LPS, whereas IL-10 expression, an anti-inflammatory cytokine, was not potentiated in 24 mo animals (Fig. 2). For IL-1β and IL-6, 24 mo animals showed a greater increase in cytokine expression at 10 ng/ml ($p < .01$) and 100 ng/ml ($p < .001$) LPS compared to 3 mo animals. IL-10 expression was significantly higher in 24 mo animals compared to 3 mo animals ($F = 10.94$, 1, 30, $p < .01$) and LPS increased IL-10 expression irrespective of age ($F = 4.54$, 4, 30, $p < .01$).

3.3. 24 mo animals exhibit increased expression of hippocampal microglia activation markers

To confirm prior reports that microglia activation markers are up-regulated with aging, we assessed the expression level of 3 commonly measured antigens.

Expression of CD11b ($F = 74.41$, 1, 30, $p < .0001$), Iba-1 ($F = 37.09$, 1, 30, $p < .0001$) and MHCII ($F = 42.13$, 1, 30, $p < .0001$) was significantly increased in 24 mo old animals compared to 3 mo animals (Fig. 3). The main effect of LPS was not significant for any activation marker.

4. Discussion

When exposed to a pro-inflammatory immune challenge, aged animals exhibit an exaggerated neuroinflammatory response. It is known that aging increases various microglial activation markers in the hippocampus (Frank et al., 2006a) as well as the hippocampal IL-1β response to both peripherally (Barrientos et al., 2009) and centrally administered LPS (Huang et al., 2008). However, these findings do not conclusively document that aging exaggerates the microglial inflammatory response to stimulation. Even if microglia were the sole source of IL-1β in the CNS, a risky assumption, it could be that in the above studies administered LPS acted at some other cell type to deliver an exaggerated signal to the microglia in the aging subjects. In the present study, hippocampal microglia from 24 mo animals showed a potentiated pro-inflammatory cytokine response to LPS suggesting that aging-induced sensitization of microglia does occur and so may underlie the potentiated neuroinflammatory responses observed in aged animals. Here, there were no other cell types for LPS