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Inflammation and oxidative stress induced by cigarette smoke in Lewis rat brains

A. Khanna [a], M. Guo [a,b], M. Mehra [c], W. Royal III [b,d,e,*]

[a] Department of Pathology, The University of Maryland School of Medicine, Baltimore, MD, United States
[b] Department of Neurology, The University of Maryland School of Medicine, Baltimore and the Maryland Center for Multiple Sclerosis Treatment and Research, MD, United States
[c] Brigham and Women's Hospital and Harvard Medical School, Boston, MA, United States
[d] The Baltimore VA Medical Center, United States
[e] Multiple Sclerosis Center of Excellence-East, United States

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ABSTRACT

Exposure to cigarette smoke has been associated with an increased risk of neurological diseases such as stroke, Alzheimer's disease and multiple sclerosis. In these studies, serum and brain sections from Lewis rats or those exposed to cigarette smoke and control rats were examined for evidence of increased inflammation and oxidative stress. Immunocytochemical staining of brain sections from CS-exposed rats showed increased expression of class II MHC and, in ELISA, levels of IFN-gamma and TNF-α were higher than for non-exposed rats. In polymerase chain reaction assays there was increased interferon-gamma, TNF-α, IL-1α, IL-1β, IL-23, IL-6, IL-23, IL-17, IL-10, TGF-β, T-bet and FoxP3 gene expression with CS exposure. There was also markedly elevated MIP-1α/CCL3, less prominent MCP-1/CCL2 and no elevation of SDF-1α gene expression. Analysis of samples from CS-exposed and control rats for anti-oxidant expression showed no significant difference in serum levels of glutathione and, in brain, similar levels of superoxide dismutase and decreased thioredoxin gene expression. In contrast, there was increased brain gene expression for the pro-oxidants iNOS and the NADPH components NOX4, dual oxidase 1 and $p22^{phox}$. Nrf2 expression, which is typically triggered as a secondary response to oxidative stress, was also increased in brains from CS-exposed rats with nuclear translocation of this protein from cytoplasm demonstrated in astrocytes in association with increased expression of the aryl hydrocarbon receptor gene, an Nrf2 target. These studies, therefore, demonstrate that CS exposure in these animals can trigger multiple immune and oxidative responses that may have important roles in the pathogenesis of CNS inflammatory neurological diseases.

1. Introduction

Links between cigarette smoking and neurological complications have been demonstrated in disease states such as stroke and Alzheimer's disease, and more recently in MS (Sundstrom et al., 2008; Cataldo et al., 2010; Shah and Cole, 2010). Underlying these associations are animal studies which show that smoking can increase levels of circulating proinflammatory markers and markers of oxidative stress, including levels of reactive oxygen species (ROS), and decrease levels of antioxidants (Churg et al., 2002; D'Hulst et al., 2005; Moerloose et al., 2005; Khanna et al., 2009). Among studies performed in humans are those which have shown that urinary levels of nucleic acid and lipid oxidative products were increased with smoking and that serum and urine levels of superoxide dismutase (SOD) and glutathione peroxidase (GPx) correlated inversely with levels of the nicotine metabolite cotinine in smokers (Harman et al., 2003; Sobczak et al., 2004). In addition to activating proinflammatory responses and cellular stress, CS and nicotine have effects that can result in immunosuppression (Geng et al., 1995; Sopori and Kozak, 1998; Sopori et al., 1998; Chen et al., 2007). Therefore, using a Lewis rat model we examined these possible effects of cigarette smoke on the expression of markers of proinflammatory and anti-inflammatory responses. We also examined the effects on the expression of NADPH oxidase, pro-oxidant and antioxidant genes and on the activation of Nrf2. These studies showed that a number of these responses increased in brains of rats with significant CS exposure. This study, which represents the first demonstration of these effects from CS in the brain, provides a basis for future investigations of the specific mechanisms by which cigarette smoking may trigger the development of inflammatory and degenerative nervous system diseases.

2. Materials and methods

2.1. Exposure of rats to cigarette smoke

This study was conducted according to the Guidelines for Animal Experiments of the NIH using CS-exposed and non-exposed Lewis rats (Harlan) (n = 8 rats/group). Rats were exposed to cigarette

* Corresponding author at: Neurology and Anatomy and Neurobiology, University of Maryland School of Medicine, 110 South Paca Street, 3rd Floor, Baltimore, MD 21201, United States. Tel.: +1 410 328 5605; fax: +1 410 328 5425.
E-mail address: wroyal@som.umaryland.edu (W. Royal).

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smoke in a specially constructed smoke chamber where the animals were restrained and ventilated by a smoking machine (Khanna et al., 2009). In the machine, smoke is sucked from lit cigarettes by syringes and pumped around a chamber, where the rats inhale the cigarette smoke. The smoking machine was set to inhale and exhale at intervals mimicking human smoking with each cigarette lasting 8–10 min. 3R4F research grade cigarettes with a regular amount of nicotine were used in this study (KTRDC Tobacco Biotechnology Group, University of Kentucky, USA). Animals were exposed to cigarette smoke 5 days/week for a total of six weeks with four cigarettes given intermittently throughout the day. Control rats were placed in smoking chambers four times a day for a total period of time that was the same as for exposed rats, but control rats were not exposed to cigarette smoke. The rats were under constant supervision to ensure that the animals did develop signs of distress. The efficacy of cigarette smoke exposure was assessed by quantification of levels of cotinine, the metabolite of nicotine, in serum samples from the rats in an ELISA (Calbiotech). All procedures were approved by the University of Maryland, Baltimore Animal Care and Use Committee.

2.2. Detection of mRNA by real time PCR

RNA samples were isolated from brain tissues using a kit from Promega (Madison, USA) and reverse-transcribed into cDNAs by using a cDNA synthesis kit from Invitrogen (Carlsbad, CA). Primer sequences utilized for the PCR reactions are listed in Table 1; RORC primers were purchased from SABiosciences. Real-time quantitative RT-PCR was performed using a Bio-Rad iCycler system (Bio-Rad). For each gene the mRNA expression measured in each sample was normalized to that for β-actin mRNA, and relative mRNA gene expression versus the internal control was calculated as previously described (Khanna et al., 2009).

2.3. Quantitation of glutathione levels

Glutathione levels were measured in serum from the exposed and non-exposed rats using the GSH-Glo™ Assay (Promega) according to the directions provided by the manufacturer.

Table 1
List of PCR primers.

Gene name	Sense (5′→3′)	Anti-sense (5′→3′)
IL-4	TGATGTACCTCCGTGCTTGA	GTGAGTTCAGACCGCTGACA
IL-6	TCAAGGGAAAAGAACCAGACA	GGTTTCAAATCACTCACCCATAC
IL-10	AGTGGAGCAGGTGAAGAATGA	TCATGGCCTTGTAGACACCTT
IL-1α	AGTTTCAATCAGCCCTTTACTGA	CTGGGTTGGGATGGTCTCTTC
IL-1β	TGTGATGAAAGACGGCACAC	CTTCTTCTTTGGGTATTGTTTGG
TNF-α	GGTCTGAGTACATCAACCTGGA	GTCTGTGCCCACATGTTCC
TGF-β	CCTGCCCCTACATTTGGA	TGGTTGTAGAGGGCAAGGAC
IFN-γ	CCACTATGGATCAGGGAAGG	TCACAATGATTCCACCCACA
IL-17	CTTCACCCTGGACTCTGAGC	CCTCAGCGTTGACACAGC
IL-18	GCCTGATATCGACCGAACA	CCTTCCATCCTTCACAGATAGG
SDF-1α	GCGCTCTGCATCAGTGAC	GTTGAGGATTTTCAGATGTTTGAC
Tbet	GCGCCAGGAAGTTTCATTT	CATTCTGGTAGGCAGTCACG
FoxP3	AAGTGACGTGCCCCGTATC	TCCGAGTCCAGTAGGTGCTT
GATA-3	AAGGCATCCAGACCAGAAAC	GTTAAACGAGCTGTTCTTGGG
MCP-1	AGCATCCACGTGCTGTCTC	GATCATCTTGCCAGTGAATGAG
MIP-1α	GCGCTCTGGAACGAAGTCT	GAATTTGCCGTCCATAGGAG
$p22^{phox}$	GCCATTGCCAGTGTGATCTA	AATGGGAGTCCACTGCTCAC
iNos	ACCATGGAGCATCCCAAGTA	CAGCGCATACCACTTCAGC
NADPH oxidase 4 (NOX4)	GCTTACCTCCGAGGATCACA	TCTGCTTTTATCCAACATCTCC
Dual oxidase 1 (DUOX1)	CACCTCCTGGAGACCTTTTC	CCCTTTGTAGCTTGGGGTTC
Superoxide dismutase	CTGGACAAACCTCAGCCCTA	CTGGACAAACCTCAGCCCTA
Thioredoxin	ATGGCCACACTTTTCTGGAC	ATGGCCACACTTTTCTGGAC
Arylhydrocarbon receptor	CTGCTTCATTTGTCGTGTCC	TTTCCTTGGAACTGCATAGTCA
β-actin	CCCAGCACAATGAAGATCAA	CGATCCACACGGAGTACTTG

2.4. Enzyme-linked immunosorbent assays

Brain tissue lysates (100 μl volumes) from each TG and WT rat were prepared and analyzed in duplicate using commercial ELISA kits for levels of IFN-γ (BD Biosciences), TNF-α (BD Biosciences) and IL-1β (R&D Systems) as previously described (Royal et al., 2012).

2.5. Tissue staining

Immunoperoxidase staining of 10 μm sections of frozen tissue fixed for 10 min in 4% paraformaldehyde was performed using Vectastain kits (Vector Labs) with a 1:200 dilution of anti-class II MHC antibodies (Pierce) according to the kit directions. For immunofluorescence staining the sections were fixed in 100% methanol (Fisher) for 10 min and then the slides were washed and then blocked with blocking solution, then incubated with the following primary antibodies: rat anti-GFAP (1:50 dilution; BD Biosciences), mouse anti-TNF-α (1:50 dilution; BD Biosciences) and mouse anti-Nfr2 (1:200 dilution; kind gift of Dr. Anil Jaiswal, University of Maryland). After incubations at 4 °C overnight with an appropriate primary antibody, the sections were washed and then incubated with, as appropriate, either fluorescein isothiocyanate (FITC)-conjugated (Santa Cruz Biotechnology) or Texas Red-conjugated (Invitrogen) secondary antibodies. For all staining, the specificity of the procedures was verified by negative control sections incubated without primary antibody. Imaging was performed using a Zeiss Axiovert 40 microscope equipped with an AxioCam MRc fluorescence camera (Zeiss) and AxioVision LE software (Zeiss) with subsequent image processing performed using SPOT Imaging software (Spot Imaging Solutions).

2.6. Statistical analyses

Relative gene expression levels were compared using the Mann–Whitney test and glutathione levels were compared using the Student's t-test. A p-value <0.05 was considered statistically significant.

3. Results

3.1. Cigarette smoke induced neuroinflammation detected in immunostained brain sections from exposed rats

Sections were stained for MHC class II, which is expressed by T cells, B cells, monocytes, macrophages, and, in the brain, also by microglia and astrocytes. These studies showed increased MCH class II staining in parenchymal and in perivascular regions of brain from the CS-exposed rats (Fig. 1). In contrast, no staining was noted in brain tissue from the control rats. Lysates of brain tissue from the CS-exposed and non-exposed rats were also examined by ELISA for levels of the proinflammatory cytokines IFN-γ and TNF-α. These studies showed that levels of these cytokine were increased for the rats exposed to CS as opposed to non-exposed rats (Fig. 2).

3.2. Cigarette smoke exposure induces the expression of inflammatory marker genes

PCR assays were performed to measure brain expression of proinflammatory and anti-inflammatory cytokines and chemokines and of transcription factors that induce the differentiation of proinflammatory and anti-inflammatory T cell subtypes. These studies showed significant upregulation of the IFN-γ, TNF-α and IL-1β (Th1) cytokine gene expression. In addition, there was increased expression of IL-1-α, IL1-β, and of Th17 (IL-23, IL-6, IL-17), Th2 (IL-10) and Treg cell (TGF-β and IL-10) associated cytokine gene expression (Fig. 3). Notably, however, IL-4 gene expression was not significantly elevated for CS-exposed versus control rats. Expression of the GATA-3 gene, which activates expression of IL-4, and RORC, which induces Th17 cell