Anti-inflammatory effects of 8-hydroxydeoxyguanosine in LPS-induced microglia activation: suppression of STAT3-mediated intercellular adhesion molecule-1 expression

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Abbreviations: ChIP, chromatin immunoprecipitation; ICAM-1, intercellular adhesion molecule-1; NAC, N-acetyl-cysteine; oh⁸dG, 8-hydroxydeoxyguanosine; oh⁸Gua, 8-hydroxyguanine; ROS, reactive oxygen species; STAT3, signal transducer and activator of transcription 3

Abstract

To elucidate the roles of 8-hydroxydeoxyguanosine (oh⁸dG), the nucleoside of 8-hydroxyguanine (oh⁸Gua), we examined the effects of oh⁸dG upon LPS-induced intercellular adhesion molecule-1 (ICAM-1) expression and the underlying mechanisms in brain microglial cells. We found that oh⁸dG reduces LPS-induced reactive oxygen species (ROS) production, STAT3 activation, and ICAM-1 expression. oh⁸dG also suppresses pro-inflammatory cytokines, such as TNF- α , IL-6 and IFN- γ . Overexpression of dominant negative STAT3 completely diminished STAT3-mediated ICAM-1 transcriptional activity.

Chromatin immunoprecipitation studies revealed that oh⁸dG inhibited recruitment of STAT3 to the ICAM-1 promoter, followed by a decrease in ICAM-1 expression. Using mice lacking a functional Toll-like receptor 4 (TLR4), we demonstrated that, while TLR4^{+/+} microglia were activated by LPS, TLR4^{-/-} microglia exhibited inactivated STAT3 in response to LPS. Evidently, LPS modulates STAT3-dependent ICAM-1 induction through TLR4-mediated cellular responses. Oh⁸dG apparently plays a role in anti-inflammatory actions via suppression of ICAM-1 gene expression by blockade of the TLR4-STAT3 signal cascade in inflammation-enhanced brain microglia. Therefore, oh⁸dG in the cytosol probably functions as an anti-inflammatory molecule and should be considered as a candidate for development of anti-inflammatory agents.

Keywords: intercellular adhesion molecule-1; microglia; reactive oxygen species; STAT3 transcription factor; Toll-like receptor 4; 8-hydroxydeoxyguanosine

Introduction

8-hydroxyguanine (oh⁸Gua) is one of the most common DNA lesions generated by reactive oxygen species (ROS) (Kasai *et al.*, 1997). To date, many studies have focused on this oxidized DNA base. However, all of these studies have focused on oh⁸Gua present in DNA and its mutagenic/carcinogenic action. This action is indicated by induction of GC $\rightarrow$ TA transversion due to mismatching with A (Shibutani *et al.*, 1991), an increase in oh⁸Gua levels in cellular DNA and urine due to various carcinogens, including (Fraga *et al.*, 1996; Nakae *et al.*, 1997) irradiation (Wilson *et al.*, 1993), pro-carcinogenic inflammatory conditions (Baik *et al.*, 1996), and a reduction in the oh⁸Gua level in cellular DNA and tissue fluids in response to chemopreventive agents (Chung *et al.*, 1992). Therefore, oh⁸Gua in DNA is not only a mediator of mutagenesis/carcinogenesis, but is also a cause of oxidative stress.

Recently, however, our previous research has indicated that the amount of oh⁸Gua in cytosol is increased in reaction to strong pathogenic infections and that oh⁸Gua can not be incorporated into DNA.

When present within DNA, oh^8Gua induces other base damage, whereas when oh^8Gua exists as a separate molecule outside of DNA, it protects normal bases and DNA under oxidation conditions (Kim *et al.*, 2006). The protective action of oh^8Gua was much stronger than the actions of well known ROS scavengers, such as manitol, DMSO, t-butyl alcohol, azide, and formate, indicating that oh^8Gua is a strong antioxidant. The strong antioxidant action of oh^8dG was also confirmed by the observation that oxidation of dichlorodihydrofluorecein by peroxynitrite is most severely inhibited by oh^8dG among antioxidants tested (Kim *et al.*, 2004). Many naturally occurring and synthetic antioxidants are known to have an anti-inflammatory effect and many of them are used for treatment or prevention of various inflammatory disorders, such as rheumatoid arthritis (Choi *et al.*, 2003), atherosclerosis (Shimosawa *et al.*, 2005), diabetic complications (Kocak *et al.*, 2000), and inflammatory bowel diseases (Dryden *et al.*, 2005). Therefore, the antioxidant property of oh^8dG should contribute to the anti-inflammatory effect for treatment of neuroinflammatory-related diseases. However, the molecular mechanisms by which oh^8dG exerts its anti-inflammation effects in brain microglial cells are largely unknown.

ICAM-1 is an inducible surface glycoprotein belonging to the immunoglobulin superfamily that is involved in a wide range of inflammatory and immune responses (Butcher *et al.*, 1996). During inflammation, ICAM-1 can be dramatically upregulated by bacterial lipopolysaccharide (LPS), phorbol esters, and inflammatory cytokines, such as $\text{TNF-}\alpha$, $\text{IL-1}\beta$, and $\text{IFN-}\gamma$. (Wahl *et al.*, 1996). The ICAM-1 promoter contains the STAT1/3 binding motif (GAS) sequence or the interferon response element (IRE). ROS, including hydrogen peroxide (H_2O_2) and superoxide, and pro-inflammatory cytokine, such as interleukin-6 (IL-6) and $\text{IFN-}\gamma$, are known to induce ICAM-1 transcription and inflammatory responses (Caldenhoven *et al.*, 1994; Kim *et al.*, 2002). The JAK/STAT signaling pathway has been reported to mediate growth factor actions, hormones, and cytokines (Ihle *et al.*, 2001). Recently, several studies have reported that JAK/STAT signaling mediates inflammatory responses in brain microglial cells (Kim *et al.*, 2003; Kim *et al.*, 2005). However, the precise roles of oh^8dG in the JAK/STAT signaling pathway and ICAM-1 expression remain to be clarified.

Microglial cells are the resident immune cells of the brain that secrete proinflammatory factors and ROS in response to immunological stimuli. Overresponsiveness or lack of regulation of microglia activation results in neurotoxic consequences (Liu *et al.*, 2003). Activated microglia are observed in

various neurological diseases, multiple sclerosis, and Alzheimer's disease (Kreutzberg *et al.*, 1996). The Toll-like receptor 4 (TLR4) is traditionally accepted as the primary (LPS) receptor (Lien *et al.*, 2000) and has been reported as critical for the microglial response to LPS (Lehnardt *et al.*, 2002). Thus, it may be functionally important to tightly regulate the degree of microglial activation after injury and during inflammatory disease.

In this study, we provide novel evidence that the anti-inflammatory actions of oh^8dG in the cytosol result from inhibition of ICAM-1 expression via the TLR4-STAT3 signal cascade and a ROS dependent mechanism in brain microglia. These findings indicate that oh^8dG in the cytosol functions as an anti-inflammatory molecule and that STAT3-dependent repression of inflammatory gene expression may be used to control an excess immune response in various inflammatory diseases. Therefore, the observed anti-inflammatory functions of oh^8dG indicate that it is a candidate for use in treatment of neuro-inflammatory diseases.

Materials and Methods

Reagents

Oh^8dG , 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and LPS were purchased from Sigma (St. Louis, MO). Antibodies to phosphorylated STAT3, total STAT3, β -actin, and ICAM-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and rat anti-OX-42 (CD11b) was purchased from Serotec. Transfection reagents Lipofectamine were products of Invitrogen Life Technologies (Carlsbad, CA). Fc Oxyburst green assay reagent was purchased from Molecular Probe, Inc.

Cell culture and MTT assay

BV2 immortalized murine microglial cells were obtained from Dr. Suck K.H. (Kyungpook National University). The BV2 cell line was grown in DMEM and supplemented with 5% fetal bovine serum. Cells were serum-starved overnight before treatment with LPS (100 ng/ml). The effect of oh^8dG on the viability of microglial BV2 cells was determined using an MTT assay.

Cytokine ELISA

The production of cytokines was determined by a cytokine-specific ELISA analysis of culture supernatants. A matched antibody pair from PharMingen (Hamburg, Germany) was used according to the manufacturer's instructions for $\text{TNF-}\alpha$, IL-6 and $\text{IFN-}\gamma$.

Animals and stereotaxic injection

The intrastriatal administration of drugs was modified from a previously described method (Heneka *et al.*, 2000; Park *et al.*, 2006). Briefly, male C57BL/6 mice (approx. weighing 22–25 g) were anesthetized by intraperitoneal (i.p.) injection with sodium pentobarbital (30 mg/kg), and placed on a stereotaxic apparatus (MyneuroLab, St. Louis, MO). Mice were injected with PBS or oh^8dG (8.0 μg in 2.0 μl of PBS) or LPS (0.5 μg in 1.0 μl of PBS) into the right striatum (stereotaxic coordinates in mm with reference to bregma: AP, +0.7; ML, +2.0; DV, -3.5) or the right hippocampus (AP, -2.7; ML, +2.5; DV, -3.0) using a 10 μl Hamilton microsyringe with a 26-gauge needle. oh^8dG was administered by intrastriatal or hippocampal injection 30 min before LPS injection. Control mice received an equal volume of PBS vehicle. The incision was cleaned with saline and sutured. All surgical and experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) in the College of Medicine, Seoul National University.

Immunohistochemical analysis

After deep anesthesia, the mice were perfused with fresh 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed and postfixed at 4°C overnight, transferred to 30% sucrose in PBS for 48 h, and then coronal sections (30 μm thickness) were prepared on a cryostat. The immunohistochemical analysis of floating sections was performed as previously described (Park *et al.*, 2006). Briefly, sections were incubated for 30 min with 3% H_2O_2 in 0.1 M PBS (pH 7.4), then blocked with a solution containing 5% normal goat/or horse serum, 2% BSA, 2% FBS, and 0.1% triton X-100 for 2 h at room temperature (RT). The sections were incubated overnight at 4°C with mouse anti-phospho STAT3 (1:500), mouse anti-ICAM-1 (1:500; Santa Cruz biotechnology, Santa Cruz, CA), or rat anti-OX-42 (CD11b) (1:50; Serotec). The sections were then incubated with biotinylated secondary antibodies (1:200; Vector Labs., Burlingame, CA) for 1 h at RT, followed by incubation with an avidin and biotinylated HRP complex (1:200; Vector Labs., Burlingame, CA) for 1 h at RT. All sections were visualized with 3, 3'-diaminobenzidine. The sections were mounted onto gelatin-coated slide glass, and coverslips were applied with permount. Images were captured using an image system (KAPPA Image-Base DX 30, KAPPA Opto-electronic Inc). For immunofluorescence staining, floating sections were incubated overnight at 4°C with a mixture of mouse anti-phospho STAT3 antibody (1:500) and rat anti-OX-42 antibody (1:50). The sections were then

incubated for 1 h at RT with a mixture of TRITC- and FITC-conjugated mouse/rabbit IgG antibody (1:200; Jackson ImmunoResearch). The sections were mounted with VectaShield (Vector Labs., Burlingame, CA). The double-stained images were analyzed using confocal laser scanning microscopy (LSM 5 PASCAL; Carl Zeiss, Germany).

ROS production assay

BV2 cells were plated at 2×10^6 cells/ml and serum-starved overnight. The Green assay reagent (Molecular Probes) consisted of bovine serum albumin (BSA) that was covalently linked to dichlorodihydrofluorescein (H_2DCF) and complexed with purified rabbit polyclonal anti-BSA IgG antibodies. Before initiating the reaction by adding Fc-Oxyburst, BV2 cells were preincubated with each reagent, a potent antioxidant compound, N-acetyl-cysteine (NAC), and oh^8dG for 1 h. Fc-Oxyburst immune complexes (120 $\mu\text{g}/\text{ml}$) were then added followed by incubation for 30 min at 37°C with LPS. Fluorescence was measured using FACSCaliber flow cytometry (Becton Dickinson, Mountain View) at 530 nm.

Western blot analysis

Cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with primary antibodies and peroxidase-conjugated secondary antibodies (Zymed, San Francisco, CA) and then visualized using an ECL system.

RT-PCR

Cellular RNAs were extracted using TRIZOL reagents (Invitrogen, Carlsbad, CA). RT-PCR was performed in a final volume of 25 μl using a Super Script One-step RT-PCR kit (Invitrogen, Carlsbad, CA) in an automated thermal cycler (GeneAmp PCR system 9700, Perkin Elmer, Norwalk Ct). PCR assays were performed over 32 cycles consisting of denaturation at 94°C for 30 s, annealing at 56°C for 40 s, and extension at 72°C for 1 min. PCR products were analyzed in 1.7% agarose gel containing ethidium bromide (EtBr). The sequences of PCR primers were reverse, 5'-CAGGGAAGAATCTGG-AAAGGT--3', and forward, 5'-AAATGGGCTTTCCG-AATTCA--3', for TNF- α ; reverse, 5'-AAGTGCATCA-TCGTTGTTCATACA--3', and forward, 5'-GAGGAT-ACCACTCCCAACAGACC--3', for IL-6; reverse, 5'-AAATTAGTTATGAATCCTTTCTGTCATC-3', and forward, 5'-ACAACACCATGGGCCCCGAGGCGGC-3', for IFN- γ .

Luciferase reporter assay

Dominant-negative mutants of the STAT3 and ICAM-1 luciferase reporter constructs were obtained from Dr. M Shong (Chungnam National University). Transient transfections were performed in duplicate in 35 mm dishes using Lipofectamine reagents as instructed by the manufacturer (Invitrogen Life Technologies). To normalize for variations in cell number and transcription efficiency, all clones were co-transfected with pCMV- β -galactosidase for 24 h. A luciferase assay was performed according to the manufacturer's instructions (Promega, Madison, WI) using 20 μ l of cell extract in 100 μ l of assay buffer. The light intensity was measured for 30 s on a Luminometer (Berthold Lumat LB9501; Berthold Technologies, Oak Ridge, TN). The luciferase activity was normalized by measurement of the β -galactosidase activity (in OD⁴²⁰).

Chromatin immunoprecipitation assay

A chromatin immunoprecipitation (ChIP) assay was performed as described previously (Ye *et al.*, 2001). oh⁸dG treated BV2 cells were fixed in formaldehyde. Soluble chromatin samples were immunoprecipitated with anti-STAT3 antibody at 4°C for 3 h. DNA isolated from immunoprecipitated material following reversal of formaldehyde crosslinking was amplified by PCR. Promoter-specific primers included mouse ICAM-1, 5'-ACTCACCTGCTGGTCTCTGA-3' for sense

and 5'-CGCTTTTATAGTCTCTGGCT-3' for antisense, which amplified 230 bp fragments flanking the STAT3 binding element.

Statistical analyses

Statistical analyses were performed using Student's t-test. Data were considered statistically significant when *P* was less than 0.05.

Results

Brain BV2 microglial cells with oh⁸dG show almost no change in viability

To determine the toxicity of oh⁸dG, we first examined cell viability using the MTT test in BV2 cells. After 72 h of incubation, cell viability was determined. As shown in Figure 1A, the viability of BV2 cells was not affected by oh⁸dG (0-100 μ g/ml). After confirmation of the pharmacologic effects, a toxicological study of oh⁸dG was performed to examine possible side effects.

Oh⁸dG inhibits LPS-elicited production of proinflammatory cytokines in microglial BV2 cells

In order to determine the functional significance of the oh⁸dG-mediated decrease in proinflammatory cytokines, oh⁸dG-treated BV2 cells were prepared

Figure 1. Intrastratial oh⁸dG administration attenuates LPS-induced inflammatory responses in the brain. (A) Examination of oh⁸dG toxicity in BV2 brain microglial cells by MTT assay. BV2 microglial cells were treated with various concentrations of oh⁸dG (0-100 μ g/ml) for 3 days and cell viability was examined by MTT assay. (B) Effect of oh⁸dG on the expression of pro-inflammatory cytokines in BV2 cells. Cytokines in the culture supernatants were analyzed by ELISA. Values represent mean $\pm$ S.E.M. (*n* = 5). #*P* < 0.01 vs. medium alone-treated group. **P* < 0.01 vs. LPS alone-treated group. (C) IL-6, TNF- α , and IFN- γ mRNA levels were also analyzed to determine the effects of oh⁸dG in cytokine production. The transcription of β -actin was measured for normalization.

and examined. The cells were treated with LPS alone or with various concentrations of oh^8dG for 24 h. The concentrations of cytokines in the culture supernatant of BV2 cells were measured by ELISA. As shown in Figure 1B, the amounts of pro-inflammatory cytokines, such as $TNF-\alpha$, IL-6, and $IFN-\gamma$ were increased in the culture medium after LPS stimulation but these increases were suppressed by oh^8dG in a dose-dependent manner. RT-PCR analysis showed that oh^8dG suppressed LPS-in-

duced IL-6, $TNF-\alpha$ and $IFN-\gamma$ mRNA expression (Figure 1C). Thus, oh^8dG also inhibits the release of pro-inflammatory cytokines, thus contributing to efficient prevention of inflammation.

Oh^8dG attenuates LPS-induced brain inflammation

A mouse model of brain inflammation due to intrastriatal (i.s.) injection of LPS (0.5 $\mu g/1.0 \mu l$ in PBS) was established to evaluate the anti-inflam-

Figure 2. Intrastriatal oh^8dG administration suppresses LPS-induced microglia activation. (A) Immunostained cortical sections 1 day after intrastriatal injections of saline + saline (a, d, g), saline + 1.0 μg of LPS (b, e, h), or 8.0 μg of oh^8dG + 1.0 μg of LPS (c, f, i). LPS-induced ICAM1 (a-c), p-STAT3 (d-f) activation and OX-42 (g-i) were markedly suppressed by intrastriatal oh^8dG administration. Photomicrographs show the marked regions (rectangles) of the insets at the right, bottom. Bar = 50 μm . (B) BV2 cells grown in 96 well plates were treated with LPS and the Fc-oxyburst immune complex in the absence and presence of 1 h pre-added oh^8dG , NAC. The fluorescence values were measured using FACS Calibur flow cytometry. Values are mean $\pm$ S.E.M. ($n = 4$). $^{\#}P < 0.01$ vs. medium alone-treated group. $^*P < 0.01$ vs. LPS alone-treated group. (C) BV2 cells were pretreated with oh^8dG , NAC, for 1 h, and then stimulated with LPS for 1 h. Cell lysates were separated by 8-10% SDS-PAGE, and western blots were probed with anti-phospho-STAT3 and STAT3.

matory effects of oh^8dG , which was administered at a dose of $8.0 \mu\text{g}/2 \mu\text{l}$ in PBS 1 h prior LPS-i.s. injection. The i.s. injection of LPS resulted in inflammation in the forebrain cortex and striatum, which was comparable to the cortex and striatum inflammation induced by i.s. injection of LPS in mice as described by Heneka *et al.* (2000). The level of brain inflammation was assessed after 1 day of

LPS-i.s. injection (Figure 2A). Figure 2A shows changes in expressions of inflammatory markers (ICAM-1). PBS- or oh^8dG -treated mice showed resting, ramified microglia-like cells with fine delicate processes that were marked by ICAM-1, p-STAT3, and OX-42 antibodies (Figure 2A-a, d, g). In LPS-treated mice, ICAM-1, p-STAT3, and OX-42-immunoreactive (IR) cells were detected in the cortex (Figure 2A-b,

Figure 3. Effects of oh^8dG on the promoter activity of ICAM-1 in BV2 cells. (A) Schematic representation of the ICAM-1 reporter genes used. (B, C) The ICAM-1 luciferase construct was cotransfected with wild type STAT3 (WT-STAT3), dominant negative STAT3 (DN-STAT3), or vector alone into BV2 cells for 24 h. The cells were pretreated with oh^8dG , NAC for 1 h, and then stimulated with LPS for 24 h. The luciferase activities shown in this study were calculated following measurement of the β -galactosidase activity to normalize for transfection efficiency. Bars represent the average luciferase activity calculated from four independent experiments. (D, E) ChIP assay. Soluble chromatin preparation from BV2 cells after oh^8dG treatment caused immunoprecipitation with the STAT3 antibody. Purified DNA was amplified by semiquantitative PCR using primers spanning the ICAM-1 promoter sequences. Five-fold dilutions of total BV2 DNA (Input) were amplified as an internal control for PCR.

e, h) and the striatum (data not shown) in the ipsilateral hemisphere. oh^8dG clearly reduced the number of ICAM-1, p-STAT3, and OX-42-IR cells (Figure 2A-c, f, i). These results indicate that oh^8dG has an anti-inflammatory effect in brain microglia.

Oh^8dG reduces LPS-induced ROS production

To study the effect of oh^8dG on ROS production, we measured the release of ROS using Fc-oxyburst. Release of ROS can be detected approximately 30 min after LPS treatment in BV2 microglia, and cells were treated with oh^8dG and NAC 1 h before LPS

exposure. As shown in Figure 2B, oh^8dG reduced LPS-induced ROS production, similar to results with NAC. Therefore, there exists a possibility that the inhibition of ROS production by oh^8dG may be due to the ROS scavenging action of oh^8dG . To further clarify that these inhibitory effects occur via STAT3 phosphorylation, we also examined the activation of STAT3. Effects similar to oh^8dG for STAT3 were observed in BV2 microglial cells (Figure 2C). This indicates that oh^8dG reduced the activation of STAT3, suggesting that STAT3-dependent inflammatory signaling is related to the anti-inflammatory activity of oh^8dG .

Figure 4. LPS-induced inflammation was mediated by TLR4. (A-D) Immunostained sections 1 day after intra-hippocampal injections of 0.5 μ g of LPS in the wild type (A, C) and TLR 4 knock-out mice (B, D). Numbers of OX-42 and p-STAT3 cells were significantly increased in the hippocampus of wild type mice, but not TLR4 knock-out mice. Astrocytes and GFAP IR cells were also significantly activated in the hippocampus of LPS-injected wild type mice (F) compared with E, however astroglial activation was notably attenuated by TLR4 deletion (G). Scale bar = 50 μ m.

Oh⁸dG suppresses LPS-induced ICAM-1 promoter activity via STAT3

We examined the possibility that ICAM-1 expression is controlled by STAT3-dependant transcriptional regulation. Figure 3A shows a schematic diagram of the deletion mutant constructs indicating the location of GAS, Sp-1, and TATA box sites. Mutation of the GAS sequence, pCAM-175 GAS (m) (see Figure 3A), eliminated LPS-dependent promoter activity (Figure 3B). To evaluate the contribution of STAT3 mediation of cellular signaling in activated microglia, we established dominant-negative mutant cells transiently expressing dominant negative (DN) STAT3. Oh⁸dG inhibited LPS-enhanced ICAM-1 promoter activity, and NAC also reduced promoter activity, indicating that ICAM-1 gene expression is STAT3 and ROS dependent (Figure 3C). We also evaluated the *in vivo* effects of oh⁸dG on the interaction of STAT3 and the ICAM-1 promoter region. A ChIP assay was performed using cells that were treated for 12 h with either LPS or oh⁸dG. Results shown in Figure 3D and E demonstrate that the amount of the ChIP complex was dramatically reduced by oh⁸dG. Thus, oh⁸dG inhibits LPS-induced ICAM-1 expression and STAT3 binding activity in BV2 cells.

LPS-induced microglia activation in the absence of TLR4

To investigate the inflammatory process in the LPS-injected brain, the activations of microglia, STAT 3, and astrocytes in the hippocampus were analyzed using immunostaining. Anti-OX-42, a marker for microglia/macrophage lineage cells, was used for microglia, anti-phospho-STAT 3 (p-STAT 3) was used for STAT 3, and anti-GFAP, a marker for astrocytes, was used for astrocytes. In LPS-injected wild type mice, the number of OX-42 and p-STAT 3 IR cells (the intensely activated form with an enlarged cell body and much shorter and thicker) was remarkably increased in both the CA1 and CA3 regions of the ipsilateral hippocampus (Figure 4A, C). OX-42 and p-STAT 3 IR cells were hardly detected in the hippocampus of LPS-injected TLR4 knock out mice (Figure 4B, D). Immunostaining for GFAP was used as a positive control for astrocytes. GFAP IR cells were also significantly activated in the hippocampus of LPS-injected wild type mice (Figure 4F, compared with Figure 4E), however astroglial activation was notably attenuated by TLR4 deletion (Figure 4G). Therefore, LPS-induced microglial activation is probably mediated, at least in part, by TLR4-STAT3 dependent responses. While several other potential LPS receptors and pathways exist (Perera *et al.*, 2001), future research will need to focus on the

effects of oh⁸dG in TLR4-dependent pathways.

Discussion

While the harmful effects of oh⁸Gua present in DNA have been studied extensively, there have been few reports about the effects of oh⁸Gua on cellular functions in the cytosol. Therefore, we were interested in the effects of oh⁸dG because cell membranes are permeable to this nucleoside and the effects of oh⁸dG can be evaluated both *in vitro* and *in vivo*. This study, for the first time, reveals that the anti-inflammatory actions of oh⁸dG are related to inhibition of STAT3 activation, a mediator involved in LPS signaling and inflammation. We have demonstrated that oh⁸dG, as an anti-oxidant, inhibits ROS production and secretion of inflammatory cytokines in LPS-stimulated microglial cells. oh⁸dG can be developed as a potent anti-inflammatory agent that can inhibit ICAM-1 expression in inflammatory diseases.

A number of studies have reported that 8-hydroxyguanine (oh⁸Gua), one of the most common DNA lesions generated by ROS, causes mutagenic/carcinogenic effects. However, our previous observations indicate that oh⁸dG is an antioxidant, based on easy degradation of oh⁸dG compared to normal nucleosides (dA, dG, dC, dT). oh⁸dG was rapidly degraded when exposed to various ROS, indicating that oh⁸dG easily interacts with ROS and is an efficient ROS scavenger. oh⁸Gua also has different functions when present in DNA and when not contained within DNA. Although oh⁸Gua is harmful when present in DNA, it can be beneficial when it exists as a separate molecule. Oh⁸Gua within DNA is rapidly released by 8-oxoguanine glycosylase 1 (OGG1). This released oh⁸Gua protects inter-molecular DNA from oxidative stress, acting as antioxidant when it exists as a separate molecule (Kim *et al.*, 2004). Antioxidant agents have anti-inflammatory effects because ROS, produced during inflammatory action by phagocytic immune cells, cause the death of pathogens (Ahluwalia *et al.*, 2004). In agreement with studies of ROS production, oh⁸dG abolishes LPS-elicited ROS production in a dose-dependent manner (Figure 2B). These inhibitory effects were also observed following addition of NAC, which markedly suppressed LPS-induced pro-inflammatory cytokine release (Figure 1B, C), STAT3 activity, and expression of ICAM-1 (Figure 2A). ICAM-1 with its counter receptor, the lymphocyte function-associated antigen 1 (LFA-1), are known to play key roles in inflammatory processes and in T-cell mediated host defense mechanisms.

The ICAM-1 promoter contains a signal transducer and activator of transcription [1(STAT1)/STAT3 binding motif (GAS sequence)] that flanks the specificity

protein 1 (Sp1) binding site, and a promoter *cis*-acting sequence (TATA) box. The JAK-STAT cascade is a regulator of various genes involved in immune and inflammatory responses (Stratowa *et al.*, 1995). Although STAT proteins were discovered during the analysis of interferon signaling, recent studies have revealed that STAT signaling can account for various cellular responses to a number of cytokines, growth factors, and hormones (Wu *et al.*, 1997). ROS, H₂O₂ and superoxide and pro-inflammatory cytokines, such as IL-6 and IFN- γ , are known to induce transcriptional complexes that bind to the ICAM-1 GAS sequence (Simon *et al.*, 1998). Mutation or deletion of this element decreases ICAM-1 promoter activity (Caldenhoven *et al.*, 1994). The Sp1-STAT3 complex appears to play an important role in upregulation of ICAM-1 transcription after reoxygenation or reperfusion (Yang *et al.*, 2005).

STAT3 was first identified as a factor in the acute phase reaction (Akira *et al.*, 1994). After induction by IL-6, STAT3 acts as a transcriptional factor for many pro-inflammatory genes, including ICAM-1 (Heinrich *et al.*, 2003). We also demonstrated that oh⁸dG suppresses the transcriptional level of ICAM-1 through STAT3 (Figure 3B, C). Our results have demonstrated that ICAM-1 expression is perfectly suppressed in DN-STAT3 expressing cells, indicating that STAT3 is an important molecule for ICAM-1 transcriptional expression. We also evaluated the *in vivo* effects of oh⁸dG on the interaction of STAT3 with the ICAM-1 promoter regions (Figure 3D, E). The amount of this ChIP complex was dramatically reduced by oh⁸dG. Thus, STAT3 proteins probably play crucial roles in regulating ICAM-1 gene promoter activity in LPS treated brain microglial cells.

Microglia, a major immune cell population in the central nervous system (CNS), are inactive in the healthy adult CNS, but become activated in acute and chronic neurodegenerative diseases (Kreutzberg *et al.*, 1996). LPS is a common inflammogen used to activate microglia in several animal models of inflammation-mediated neurodegeneration, including both *in vivo* and *in vitro* models (Gao *et al.*, 2002). The Toll-like receptor family is a diverse group of receptors that are responsible for recognition of microglial pathogens and protein patterns. This reaction is critical to the innate immune response (Means *et al.*, 2000). TLR4 is traditionally accepted as the primary (LPS) receptor (Lien *et al.*, 2000) and has been reported to be critical for the microglial response to LPS (Lehnhardt *et al.*, 2002). It has been reported that LPS is effective in rapidly up-regulating the expression of the endothelial ICAM-1 and the vascular cell-adhesion molecule (VCAM) (Bell *et al.*,

1995). Our results show that STAT3 contributes to LPS- induced inflammatory responses and oh⁸dG inhibited LPS-enhanced STAT3 activation in brain microglia. We also observed by MMT assay that oh⁸dG was not toxic (Figure 1A), indicating that the anti-inflammatory action of oh⁸dG is due to a mechanism underlying inhibition of STAT3 inflammatory signaling. However, the side effects of oh⁸dG in the cytosol are a concern. Therefore, after confirming the pharmacologic effects, a toxicological study of oh⁸dG should be performed.

Although the signals that activate microglia *in vivo* are defined incompletely, several studies using *in vitro* models have shown that microglia respond to cytokines (IL-1 β , TNF- α and IFN- γ), microbial products, such as bacterial LPS, and certain disease-related abnormal proteins, including β -amyloid, human immunodeficiency virus (HIV)-specific gp41 and HIV-Tat, prions (Nakamura *et al.*, 2002), and heat shock proteins (Kakimura *et al.*, 2002). These factors activate their receptors and activate intracellular signaling pathway. The initial *in vivo* reports using TLR4-/- mice demonstrated a reduced susceptibility to sepsis with systemic administration of LPS (Poltorak *et al.*, 1998). Early *in vitro* work with cells derived from TLR4-/- mice demonstrated that TLR4-/- monocytes have an attenuated LPS cytokine response (Hoshino *et al.*, 1999), indicating the critical role of TLR4 in the LPS-induced production of pro-inflammatory factors. However, while TLR4 is known to initiate the effects of LPS through a common and primary pathway, several other receptors have been identified in the induced effects of LPS, and there is a clear TLR4- independent response to LPS in phagocytes (Perera *et al.*, 2001). For confirmation, the role of TLR4 in LPS-induced microglial activation was tested with mutant mice. Immunohistochemical staining of microglia with antibodies against OX-42 and STAT3 was used in brain tissue treated with LPS. Absence of functional TLR4 mice microglia did not staining with antibodies against OX-42 and STAT3. In our study, TLR4 was found to be indispensable for triggering a STAT3 pathway from LPS leading to activation of microglia.

Our results demonstrate that oh⁸dG plays a role in anti-inflammatory actions via suppression of ICAM-1 gene expression by blockade of the TLR4-STAT3 signal cascade in brain microglia. Therefore, oh⁸dG in the cytosol probably functions as an anti-inflammatory molecule that will be useful as a therapeutic agent for neurodegenerative diseases.

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