The influence of brain inflammation upon neuronal adenosine A$_{2B}$ receptors

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Abstract

Alzheimer’s disease (AD) is associated with glial activation and increased levels of pro-inflammatory cytokines. Epidemiological results suggest that anti-inflammatory therapies can slow the onset of AD. Adenosine, acting at type-2 receptors, is an effective endogenous anti-inflammatory agent that can modulate inflammation both in the periphery and the brain. We investigated changes in the expression of adenosine type-2B (A$_{2B}$) receptors and a related intracellular second messenger during chronic brain inflammation and following treatment with the non-steroidal anti-inflammatory drug flurbiprofen and its nitric oxide (NO)-donating derivative, HCT1026. Chronic infusion of lipopolysaccharide (LPS) into the 4th ventricle of young rats induced brain inflammation that was associated with microglial activation and reduced neuronal immunoreactivity for adenosine A$_{2B}$ receptors in the cortex. Daily administration of HCT1026, but not flurbiprofen, reduced microglial activation, prevented the down-regulation of A$_{2B}$ receptors and elevated tissue levels of cAMP. The results suggest that a therapy using an NO-releasing NSAID might significantly attenuate the processes that drive the pathology associated with AD and that this process may involve the activation of adenosine A$_{2B}$ receptors.

Keywords: Alzheimer’s disease, anti-inflammatory, lipopolysaccharide, microglia, rats.

whether the inflammation produced by the chronic LPS infusion was attenuated using the anti-inflammatory drugs flurbiprofen or its NO-donating derivative, HCT1026 (2-fluoro-a-methyl[1,1'-biphenyl]-4-acetic acid, 4-(nitrooxy)butyl ester). HCT1026 is a novel NSAID that has good tolerability in rats and can significantly reduce the number of activated microglia in the brain (Hauss-Wegrzyniak et al. 1998b, 1999).

Materials and methods

Subjects
Male, young (3 months old, n = 36) F-344 rats (Harlan Sprague-Dawley, Indianapolis, IN, USA) were singly housed in Plexiglas cages with free access to food and water. The rats were maintained on a 12/12-h light-dark cycle in a temperature-controlled room (22°C) with lights off at 08.00 h. All rats were given health checks, handled upon arrival and allowed at least 1 week to adapt to their new environment prior to surgery.

Materials
LPS (Escherichia coli, serotype 055:B5) was obtained from Sigma (St Louis, MO, USA). Flurbiprofen and HCT1026 were obtained from NicOx, S.A., Nice, France. [3H]PK11195 was obtained from New England Nuclear, Boston, MA, USA.

Surgical procedures
Standard procedures were used for the surgery (Hauss-Wegrzyniak et al. 1998a,b, 2002). Each rat was anesthetized with isoflurane gas and placed in a stereotaxic instrument with the incisor bar set 3.0 mm below the earbars (i.e., flat skull). The scalp was incised and retracted and holes were made at appropriate locations in the skull with a dental drill. Co-ordinates for the 4th ventricle infusions were as follows: 2.5 mm posterior to lambda, on the mid-line, and 7.0 mm ventral to the dura. An osmotic minipump (Alzet, Palo Alto, CA, USA) was model 2004, to deliver 250 nL/h was attached via a catheter to a chronic indwelling cannula that had been positioned stereotaxically so that the tip extended to the co-ordinates given above. Each minipump was prepared to inject 250 nL/h or artificial CSF (aCSF). The composition of the aCSF (in mmol/L) was 140 NaCl; 3.0 KCl; 2.5 CaCl2; 1.2 Na2HPO4, pH 7.4. The HCT1026 and flurbiprofen were freshly dissolved in the vehicle (100% DMSO) and administered (s.c.) every morning for 28 days and placed in an incubator at room temperature.

The following post-operative care was provided to all rats: betadine was applied to the exposed skull and scalp prior to closure to limit local infection and 5 mL of sterile isotonic saline were injected (s.c.) to prevent dehydration during recovery. The rats were closely monitored during recovery and kept in an incubator at temperatures ranging from 30 to 33°C (Ohio Medical Products, Air Reduction Co., Inc., Madison, WI, USA). Body weights were determined daily and general behavior was monitored for seizures. This research was conducted under the supervision, and with the approval, of the University of Arizona Institutional Animal Care and Use Committee.

Finally, in order to determine whether the NO-donating NSAID HCT1026 could directly influence A2B receptor expression, an additional group of young rats were administered HCT1026 (15 mg/kg/day, s.c.) for either 3 or 7 days and then killed for histological study. HCT1026 was supplied by NicOx, S.A. These rats were not infused with either aCSF or LPS.

Histology
Rats were anesthetized with isoflurane and prepared for histological analysis by in situ perfusion of the brain with cold saline containing 1 U/mL heparin, followed by 4% paraformaldehyde in 0.1 mol/L sodium phosphate buffer, pH 7.4. The brains were removed and the location of the cannula in the 4th ventricle was confirmed. The brains were post fixed 1 hour in the same fixative and then stored (4°C) in phosphate-buffered saline.

Immunohistochemistry for activated microglia and adenosine A2B receptors
Free-floating, serial coronal sections (40 μm) were taken by vibratome, washed in PBS (pH 7.4) and incubated in a solution of PBS containing 0.5% Triton X-100 and 5% normal goat serum (microglia) or 5% normal rabbit serum (adenosine receptors) for 1 h. The monoclonal antibody OX-6 (final dilution 1 : 400, Pharmingen, San Diego, CA, USA) was used to visualize activated microglial cells. This antibody is directed against the MHC II antigen. A2B receptors were visualized using affinity-purified anti-goat polyclonal antibodies raised against the amino terminus of the receptor peptide (final dilution 1 : 100, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The sections were incubated overnight in 5% normal rabbit or goat serum with primary antibodies directed against the specific epitopes. After washing in PBS, the sections were incubated for 1 h with the appropriate secondary antibodies. After washing in PBS, the sections were incubated for 1 h at room temperature with avidin-biotin-horseradish peroxidase macromolecule complex (Vectorstain, Elite ABC kit; Vector Laboratories, Burlingame, CA, USA). The locations of immunohistochemically defined cells were examined by light microscopy.

Double immunofluorescence staining
Two biotinylated secondary antibodies and avidin-D fluorochrome conjugates were used to stain free-floating slices for A2B receptors (Fluorescein green) and neurons (Texas Red). After washing in PBS, the sections were incubated with PBS and 5% normal rabbit serum and Avidin D solution (Avidin Biotin Bloking Kit, Vector Laboratories) for 30 min and then incubated for 2 h with the primary A2B receptor antibody at a final dilution of 1 : 25 and Biotin Bloking (Vector Laboratories). After washing in PBS, the sections were incubated for 30 min with the secondary biotinylated anti-goat antibody at the same dilution previously used. After washing, the sections were incubated for 30 min with the fluorochrome Fluorescein Avidin D FITC (Vector Laboratories); then incubated for 1 h with the primary antibody NeuN (NeuN-mouse anti-neuronal nuclei monoclonal antibody, Chemicon, Temecula, CA, USA), the secondary anti-mouse biotinylated antibody was used at the same dilution previous used. The sections were incubated with Texas Red Avidin

D (Vector Laboratories) for 20 min; after washing, the sections were mounted. Images were acquired from the double-labeled sections using a confocal laser scanning microscope (Carl Zeiss, model 510NLO-META, Thornwood, NY, USA) equipped with a krypton-argon laser.

Biochemistry
Brain samples from five rats in each of the four groups were dissected and stored (~ 70°C) for assay of [3H]PK11195 binding sites and endogenous cAMP levels. The results were analyzed by analysis of variance (ANOVA) followed by pair-wise comparisons between groups, with adjustment for multiple comparisons by Bonferroni’s correction (SigmaStat software, Jandel Scientific, San Rafael, CA, USA).

Receptor binding
Two main classes of benzodiazepine receptors exist in mammals; a high-affinity receptor found in the brain that is a component of the GABA receptor complex, and a peripheral receptor site that resides on the outer mitochondrial membrane (Stephenson et al. 1995) known as the peripheral-type benzodiazepine receptor (Rao et al. 2001). This receptor is a hetero-oligomeric complex of three proteins; their major function may be the translocation of cholesterol across the mitochondrial membranes for processing into neurosteroids (Rao et al. 2001). The isoxquinoline carbamoxide PK11195 selectively binds to this peripheral receptor site and has no affinity for the central receptor. Within the brain, PK11195 binds predominantly to activated microglia at this site (Stephenson et al. 1995; Cagnin et al. 2001; Rao et al. 2001).

[3H]PK11195 filtration binding assays (Rao et al. 2001) used samples of parietal cortex that were dissected from frozen brains and homogenized in 20 vol of ice-cold 50 mmol/L Tris-HCl (pH 7.4) buffer. Membranes were prepared for binding assays by repeated centrifugation (40 000 g for 20 min at 4°C), repeated freeze-thawing and resuspension in 10 vol of buffer. Protein content of the final suspension was determined according to the method of Lowry et al. (1951). Binding assays were performed at 4°C for 2 h in a final volume of 500 μL containing 1 nM [3H]PK11195 (specific activity, 85.5 Ci/mmol). The assay was terminated by addition of 4 mL of ice-cold buffer followed by vacuum filtration through Whatman GF/B glass microfiber filters (pre-soaked in 0.3% polyethyleneimine for at least 30 min). The filters were washed twice with ice-cold buffer, air-dried by vacuum and the radioactivity on the filters was determined by liquid scintillation spectrometry. All assays were performed in triplicate; specific binding was defined by addition of 20 μmol/L diazepam to the incubation solution. The results were analyzed by ANOVA.

cAMP levels
Samples of frozen tissue from the entorhinal cortex and temporal lobe were homogenized in 10 vol of 5% trichloroacetic acid in a glass-Teflon tissue grinder and centrifuged at 2500 g for 15 min. Protein concentration in the pellet was determined in triplicate (Lowry et al. 1951). The supernatant was extracted with 3 vol of water-saturated ether and the aqueous extract was freeze-dried and stored until assayed in duplicate using a commercial ELISA kit according to manufacturers guidelines (Assay Designs, Ann Arbor, MI, USA).

Results
Overall, chronic infusion of LPS was well tolerated by all rats. Initially after surgery, all LPS-treated rats lost only a few grams of weight. Within a few days, however, most rats had regained weight and continued to gain weight normally for the duration of the study.

Studies of brain inflammation
Immunohistochemistry for activated microglia
CSF/VEH rats had a few mildly activated microglia scattered throughout the brain (Fig. 1a) similar to our previous reports (Hauss-Wegrzyniak et al. 1998a,b, 1999). In LPS/VEH rats, immunohisto-chemical staining for MHC II (using the OX-6 antibody) revealed numerous, highly activated microglia distributed throughout the neocortex, cingulate gyrus, entorhinal cortex and hippocampus (Fig. 1b). The activated microglia were characterized by a contraction of their highly ramified processes that appeared bushy in morphology (Fig. 1c). This is the typical appearance of inflammation produced by LPS infusion and is consistent with results from previous studies (Hauss-Wegrzyniak et al. 1998a,b, 1999). LPS/HCT rats had noticeably fewer activated microglial cells that were characterized by long, thin, ramified processes indicating a lower level of activation (Figs 1d and e). Chronic treatment with flurbiprofen had a minimal effect on the level of microglial activation produced by LPS-infusion (Fig. 1f). The efficacy of flurbiprofen was clearly less than its NO-releasing derivative HCT1026 in this animal model of neuroinflammation.

Analysis of the number of [3H]PK11195 binding sites upon activated microglia in the parietal cortex found a significant main effect of group (F_{3,12} = 6.12, p = 0.015). Post hoc multiple pair-wise comparisons found that rats in the LPS/VEH and LPS/FP groups (Fig. 2) had significantly more (p < 0.05) bindings sites, i.e. activated microglia, than rats in the LPS/HCT or CSF/VEH groups, who did not differ significantly from each other.

Rats in the LPS/HCT group had significantly (F_{3,12} = 4.09, p = 0.049) increased cAMP levels within the entorhinal/temporal lobe cortex, as compared with rats in the CSF/VEH or LPS/FP groups (Fig. 3). The cAMP level within the cortex of LPS/VEH rats was highly variable and did not differ significantly from any of the other three groups.

Studies of adenosine A2B receptors
Large cells were immunoreactive for A2B receptors in layers 4 and 5 of the frontal, parietal and temporal lobe, including the entorhinal cortex, of the neocortex in CSF/VEH rats (Fig. 4a). The widespread distribution of these receptors is consistent with previous reports (Dixon et al. 1996). The
immunohistochemical and immunofluorescence staining demonstrated that A2B receptors were localized to the cell membrane across most of the soma; these immunoreactive neurons were round-shaped with a dark cell body (Figs 4b and 5). In LPS/VEH rats, immunoreactive cells were reduced in number throughout all regions examined (Fig. 4c) and had a lower degree of immunoreactivity (Fig. 4d). Administration of HCT1026 (Fig. 4e), but not flurbiprofen (Fig. 4g), to
LPS-infused rats noticeably increased both the number and degree of immunoreactivity of the large neurons. In LPS/HCT rats, many cells appeared darkly stained with elaborate interconnections and intensely stained long processes. This increased immunoreactivity was in contrast to that seen in LPS/FP rats (Fig. 4h). Daily administration of HCT1026 to young naïve rats for up to 7 days had no effect upon the distribution of A2B receptor immunoreactive cells (data not shown); the staining was identical to that shown in Fig. 4(a).

**Fig. 4** Immunohistochemistry for A2B receptors. Many large immunoreactive cells were observed in layers 4 and 5 of the frontal cortex (a and b). Chronic LPS infusion for 28 days reduced both the number and level of immunoreactivity of these cells (c and d). HCT1026 (e and f), but not flurbiprofen (g and h), treatment in the LPS-infused rats greatly increased the immunoreactivity of these cortical neurons. Immunoreactivity was distributed along the dendritic arbors (f), in contrast to the more limited distribution seen in flurbiprofen-treated rats (b). Low magnification bar = 110 μm (a, c, e, g); high magnification bar = 40 μm (b, d, f and h).

### Discussion
Chronic infusion of LPS into the 4th ventricle of young rats produced an extensive inflammatory reaction throughout the brain, particularly within the frontal neocortex, cingulate gyrus, hippocampus, subiculum and the entorhinal and piriform cortices. The inflammatory response was characterized by a significant increase in the number of reactive microglial cells as determined both qualitatively by immunocytochemistry and quantitatively by *in vitro*[^1]PK11195

[^1]: PK11195
receptors on the vascular endothelium leading to an inhibition of the secretion of inflammatory cytokines (Cronstein et al. 1996; Eigler et al. 1997).

Chronic, uncontrolled brain inflammation may underlie many neurodegenerative diseases (Akiyama et al. 2000). Mechanisms exist within the brain to attenuate or terminate its inflammatory response (Mayne et al. 2001; Ohta and Sitkovsky 2001). For example, brain inflammation is associated with increased extracellular levels of adenosine (Bouma et al. 1994) that can act via the lower affinity A2B receptors to reduce the expression of class II major histocompatibility complex (MHC II) on microglia (Fiebich et al. 1996b; Xaus et al. 1999) and increase interleukin-6 mRNA levels in astrocytes (Fiebich et al. 1996a); this cytokine inhibits the production of the pro-inflammatory tumor necrosis factor-α (TNFα, Benveniste et al. 1995).

Daily treatment with the NO-donating NSAID HCT1026 prevented a decline in A2B receptor immunoreactivity on the cortical neurons of LPS-infused rats. In contrast, in the absence of brain inflammation, up to 7 days of daily HCT1026 administration to young rats did not alter A2B receptor immunoreactivity in the cortex. The absence of an effect may also imply that the effects of the drug upon A2B receptors may require a longer treatment period. Our findings suggest that the inhibition of cyclooxygenase activity by this novel NSAID was sufficient to prevent the inflammation-induced decline in A2B receptor immunoreactivity.

Chronic neuroinflammation produced by infusion of LPS did not significantly increase cAMP levels, as compared with CSF/VEH controls. Because the production of cAMP in neurons can be increased following stimulation of A2B receptors (Lupica et al. 1990), we hypothesize the following conditions to explain the lack of inflammation-induced increase in cAMP levels (Fig. 6): the chronic LPS infusion elevates extracellular adenosine (Bouma et al. 1994) and produces a down-regulation of neuronal A2B receptors. Although other inflammatory stimuli might continue to produce cAMP, according to our model, the prolonged absence of these receptors precludes any continued inflammation-induced elevations in cAMP production. In contrast, daily therapy with the NSAID HCT1026 prevents the LPS-induced reduction in A2B receptors and allows a typical, and significant, elevation in cAMP levels that then persists for the entire treatment period. The predictions of this model are consistent with the findings of the current study and also propose a testable hypotheses that adenosine levels remain elevated in the presence of NSAID therapy and that the cAMP elevation associated with the drug treatment, in the presence of brain inflammation, is due to A2B receptor activation. Under these conditions of chronic NSAID therapy, the increased production of cAMP and adenosine by neurons and glia would also be associated with an inhibition of the production and release of the inflammatory cytokines interleukin-1α and TNFα (Bouma et al. 1994;
Neuroinflammation and \(A_{2B}\) receptors

![Fig. 6](image)

In the presence of a chronic inflammatory stimulus, extracellular cytokines and adenosine levels are elevated. The activation of NF-\(\kappa\)B within cells could be increased by the presence of IL-1 and TNF\(\alpha\). NF-\(\kappa\)B could then stimulate transcription of inflammatory genes. Adenosine, acting through its \(A_{2B}\) receptor that is coupled to \(G_s\) proteins, would increase cAMP levels via the activation of adenylyl cyclase; cAMP would then inhibit NF-\(\kappa\)B activity. This simplified cartoon demonstrates why, following the infusion of LPS, cAMP levels did not become elevated due to the reduced expression of the \(A_{2B}\) receptor. The NSAID therapy, by preventing the reduced expression of the \(A_{2B}\) receptors in the presence of elevated adenosine and by reducing the level of cytokines, would lead to increased cAMP levels.

Overall, the current study confirmed our previous findings that the novel NO-releasing NSAID HCT1026 can significantly reduce the degree and extent of brain inflammation following chronic infusion of LPS into the 4th ventricle (Hauss-Wegrzyniak et al. 1998b, 1999; Wenk et al. 2000). We have previously shown that HCT1026 therapy can reverse inflammation-induced memory impairment (Hauss-Wegrzyniak et al. 1999). The effectiveness of the NO-releasing derivative of flurbiprofen was noticeably greater than flurbiprofen alone. We speculate that the effects of HCT1026 are due to the release of NO within the blood and the subsequent cascade of a series of beneficial biochemical and immunological changes in the periphery that develops in the presence of these changes that would then act synergistically with flurbiprofen in the brain. We also speculate that the release of NO in the blood might also improve the uptake of flurbiprofen across the blood–brain barrier. Therefore, the effectiveness of HCT1026 is not thought to be due to the release of NO within the brain. The NO molecule might also produce significant changes in blood chemistry that might also influence the integrity of the blood–brain barrier. Indeed, recent evidence suggests that only flurbiprofen, and not the HCT1026 molecule as such, is actually found in the brain in measurable levels (Aldini et al. 2002). Once inside the brain, the anti-inflammatory properties of released flurbiprofen molecule are probably due to its ability to inhibit both isoforms of the cyclooxygenase enzyme leading to the suppression of prostaglandin synthesis (Minghetti and Levi 1995; Santini et al. 1996). Due to the potential significance of chronic neuroinflammation in the progression of AD (Akiyama et al. 2000), our results suggest that a therapy using an NO-releasing NSAID will significantly attenuate the processes that drive the pathology associated with AD.

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References


