Cyclooxygenase-1 in Human Alzheimer and Control Brain: Quantitative Analysis of Expression by Microglia and CA3 Hippocampal Neurons

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Abstract. Epidemiological and clinical studies suggest that nonsteroidal anti-inflammatory drugs (NSAIDs) that inhibit cyclooxygenase (COX) slow the progression and delay the onset of Alzheimer disease (AD). Two isoforms of cyclooxygenase have been identified. Although much effort has recently been focused on the inducible COX-2 isoform, little is known about COX-1 expression in human brain. We report that COX-1 message and immunoreactivity are localized to human hippocampal CA3 and CA4 neurons, granular neurons in neocortical layer IV, and occasional cortical pyramidal neurons. Quantitative in situ hybridization showed no differences between COX-1 mRNA levels in control and AD CA3 hippocampal neurons. COX-1 immunoreactivity was also present in microglial cells in gray and white matter in all brain regions examined. COX-1 appeared to be expressed in microglial cells regardless of their activation state as determined by HLA-DR immunostaining. However, COX-1 immunopositive microglia were found in association with Aβ plaques, and the density of COX-1 immunopositive microglia in AD fusiform cortex was increased. This pattern suggests an overall increase of COX-1 expression in AD. Currently used NSAIDs inhibit both isoforms of cyclooxygenase. The present study shows that COX-1 is widely expressed in human brain, and raises the possibility that COX-1 may contribute to CNS pathology.

Key Words: Alzheimer disease; CNS; Hippocampus; Human; Microglia; Nonsteroidal anti-inflammatory drugs; Prostaglandin-endoperoxide synthase.

INTRODUCTION

The progressive dementia of Alzheimer disease (AD) is associated with neuropathological changes that arise in the transentorhinal, then entorhinal cortex and hippocampal regions, and spread to include much of the neocortex and some subcortical nuclei. Together with neuronal pathology and amyloid deposition, inflammation, characterized by gliosis and production of inflammatory molecules, is an important part of AD pathology (1–3). It has been argued that tissue inflammation may be one of the mechanisms necessary to trigger neurodegeneration in AD (1, 3–6). According to this hypothesis, an underlying fundamental process such as amyloid β deposition triggers a cycle of inflammation that is maintained by local glia and results in neuronal toxicity and death.

Additional evidence supporting this hypothesis comes from clinical observations and epidemiological studies. More than 15 studies have shown that people who used nonsteroidal anti-inflammatory drugs (NSAIDs) for unrelated reasons had a decreased risk of developing AD (7, 8). These retrospective studies have been confirmed by a longitudinal study showing that the relative risk for AD decreases with increasing duration of NSAID use (9). Rogers and colleagues (10) reported that AD patients treated for 6 months with indomethacin showed slight improvement in cognitive function tests, whereas patients receiving placebo showed decline. In addition Rich et al (11) found that AD patients taking NSAIDs performed better on cognitive tests than those not taking NSAIDs. Together these studies provide substantial evidence that anti-inflammatory therapy is beneficial in AD.

NSAIDs act by inhibiting cyclooxygenase, the rate-limiting enzyme of prostanoid synthesis. Two isoforms of cyclooxygenase have been described, COX-1 and COX-2. COX-1 is constitutive in many tissues. COX-2 is an immediate early gene that can be induced by a variety of stimuli such as injury, growth factors, cytokines, and endotoxins (12–15). Cyclooxygenase-2 and prostaglandins are recognized as important mediators of inflammation in peripheral tissues (16–20). There is accumulating data that prostaglandins and COX-2 are upregulated in CNS injury and contribute to neuronal vulnerability. For example, COX-2 specific inhibitors reduce infarct volume following middle cerebral artery occlusion (21) and attenuate the loss of CA1 hippocampal neurons in a model of global ischemia (22).

Based on these findings attention has focused on the role of COX-2 in AD and clinical studies using COX-2 selective inhibitors are underway. However, all NSAIDs used to date inhibit the activity of both COX isoforms. In the current study we examined the expression of COX-1 in human brain and addressed its possible role in AD pathogenesis.
MATERIALS AND METHODS

Tissues

Human hippocampus and cortex were obtained from the Sun Health Research Institute (Sun City, AZ) and the University of Rochester Alzheimer's Disease Center (Rochester, NY) Brain Banks. Tissues from 10 clinically and pathologically diagnosed AD patients and 10 subjects without clinical symptoms of dementia and AD neuropathology were obtained from Sun City and used for all quantitative studies. Age of control patients was 74 to 92 years, 80.7 on average; postmortem delay was 1.66 to 2.75 hours (h), average 2 h. Subjects with AD were 76 to 90 years old, 81.2 on average; postmortem delay was 1.5 to 4.5 h, average 2.6 h. Tissue was immediately fixed in 4% paraformaldehyde for 24 h, then cryoprotected in 2% dimethyl sulfoxide (DMSO), 10% glycerol in phosphate buffer (PB) pH 7.4 for 48 h, followed by incubation in 2% DMSO, 20% glycerol in PB for 48 h. 40-μm-thick sections were cut on a sliding microtome and stored in cryoprotectant (33% ethylene glycol, 33% glycerol and 33% PB) at −20°C.

Human right temporal cortex biopsy tissue was obtained from 2 epileptic patients undergoing partial temporal lobectomy at Strong Memorial Hospital (Rochester, NY). Tissue was immediately immersion fixed in 4% paraformaldehyde for 4 h, then cryoprotected in 30% sucrose, and cut at 40 μm. Macaque monkey occipital cortex perfusion fixed with 4% paraformaldehyde and cut at 40 μm was a generous gift from Dr. Suzanne Huber (University of Rochester). Recently we determined that fixation of human and animal tissue in 2.5% glutaraldehyde (v/v), 1% acetic acid (v/v), 10 mM sodium-m-periodate, and 40 mM sodium phosphate, pH 4.0 (23, 24) provides better immunohistochemical visualization of COX. Rat brain tissue was perfusion fixed with 2.5% glutaraldehyde as described above and cut at 35 μm.

Antibodies

To establish antibody specificity we prepared western blots loaded with cyclooxygenase proteins and probed them with anti-COX antibodies. Purified, recombinant, human COX-2 and rat COX-1 and 2 were supplied courtesy of Dr. Phyllis Whiteley (Roche Bioscience, Palo Alto, CA). Human platelet-rich plasma obtained from the Blood Bank at the University of Rochester was used as human COX-1 positive control. The platelet-rich plasma was acidified to pH 6.5 by the addition of citric acid and centrifuged at 300 g for 15 minutes (min). The pellet containing platelets was washed twice and resuspended in western sample buffer. Protein concentration was assayed using a BCA kit (Pierce, Rockford, IL).

Proteins were subjected to 10% SDS-PAGE, and transferred to polyvinylidene fluoride membrane (Millipore Corporation, Bedford, MA) to assay antibody specificity (15). The membrane was re-wet with methanol and nonspecific binding was blocked with 2% BSA and 3% nonfat dry milk for 1 h at room temperature. Primary antibodies were applied at a concentration of 1:1,000, and allowed to incubate with agitation overnight at 4°C. The following day membranes were incubated with 1:5,000 dilution of horseradish peroxidase conjugated secondary antibodies of the appropriate species specificity (Amersham Life Science Inc., Arlington Heights, IL) for 2 h at room temperature. Signal was visualized by reaction with enhanced chemiluminescent solution (ECL: Amersham Life Science Inc.) and immediate exposure to film.

Immunohistochemistry

TL, a monoclonal antibody raised against human COX-2 (catalog no. C22420; Transduction Laboratories, Lexington, KY) was shown to cross-react with COX-1 and was used at 1: 1,000. Polyclonal antibody PG16 (Oxford Biomedical, Oxford, MI) specific for COX-1, and PG27 (Oxford Biomedical) specific for COX-2, were each used at 1:100. Anti-GFAP (1:3,000, Dako A/S, Denmark) and anti-HLA-DR (LN-3, 1:100, ICN Pharmaceuticals, Costa Mesa, CA) antibodies were used to label activated astrocytes and microglia, respectively. The biotinylated lectin, RCA-1 (1:50, E-Y laboratories, San Mateo, CA) was used to label microglia. The 10D5 anti-amylloid β antibody (Athena Neuroscience, S. San Francisco, CA) was used at 1:100 following antigen retrieval with 0.4% pepsin (Sigma, St. Louis, MO) for 1 h at room temperature.

Immunohistochemistry (IHC) of free-floating 40 μm sections was performed in net wells according to standard protocol (24). Sections were washed free of cryoprotectant in phosphate buffered saline with 0.1% Tween-20 (PBS-T), then incubated for 20 min in PBS containing 1% hydrogen peroxide and 10% methanol to inhibit endogenous peroxidase activity. Antigen retrieval was performed in 100% cold methanol for 20 min (25). Tissue was rehydrated in PBS-T, then incubated in 0.3% BSA and 2–10% serum (horse for monoclonal antibodies, goat for polyclonal) in PBS-T for 2 h to block nonspecific binding and enhance tissue permeability. Subsequently, sections were incubated in the same blocking solution containing primary antibody at 4°C with agitation for at least 12 h. Sections were washed, then incubated for 2 h in the blocking solution containing 1:500 to 1:1,500 dilution of biotinylated secondary antibody (horse anti-mouse or goat anti-rabbit; Vector, Burlingame, CA). Following a series of washes in PBS-T, sections were incubated with a 1:1,000 dilution of streptavidin-HRP (Boehringer Mannheim Corporation, Indianapolis, IN) in PBS-T for 1 h. Immunostaining was visualized with HRP substrates: diaminobenzidine (DAB, brown, Sigma), Vector SG (blue-gray, Vector) or 3-amino 9-ethylcarbazole (AEC, red, Sigma). The sections were dehydrated through alcohols and xylenes and coverslipped in Cytosil 60 (Stephens Scientific, Riverdale, NJ). AEC stain was preserved with Immu-Mount without dehydration (Shandon Lipshaw, Pittsburgh, PA).

For double labeling experiments the above protocol was repeated and a complementary colored substrate was used. Absorption controls were performed by incubating approximately 10-fold excess ovine COX-1 antigen (Oxford Biomedical, Oxford, MO) with undiluted primary antibody for 1 h before addition to the tissue. Controls omitting primary antibody were also carried out and were consistently negative.

Riboprobe Preparation

Plasmid containing a full-length human COX-1 cDNA insert (generously provided by Dr. D. A. Young, University of Rochester) was linearized with Hind III for the generation of an antisense probe and with Not I for sense probe. Since COX-1

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and 2 have regions with high homology, we prepared a human COX-1 specific probe by PCR amplification of nucleotides 134 through 486 with a T7 promoter sequence attached to the lower primer (upper primer, 20 mer 5’ GGGCATCTGTGTCGGCTTCG 3’; lower primer, 45 mer 5’ TCTAATACGACTCACTATAGGGCGAGTGGGGCCAATCTTTAGGC 3’). The PCR product was gel purified, PCR reamplified, and a NAP-5 spin column (Pharmacia Biotech AB, Uppsala, Sweden) was used to remove free nucleotides. 32P or 35S UTP labeled riboprobes were synthesized using Promega transcription components (Madison, WI). COX-1 full-length sense probe and COX-1 antisense short probe were transcribed with T7 RNA polymerase (Life Technologies, Gaithersburg, MD); COX-1 full-length antisense probe was transcribed using SP6 RNA polymerase (Life Technologies). Specific activities of 35S probes were 3 x 106 cpm/µg and 6 x 106 cpm/µg for 32P probes.

In Situ Hybridization

Sections were washed free of cryoprotectant in PBS for 45 min then mounted in water on Superfrost slides (Fisher Scientific, Pittsburgh, PA), air dried, and stored at -80°C. In situ hybridization was performed as described by Callahan et al (26). Slides were postfixed in 4% paraformaldehyde, treated with 1 µg/ml proteinase K (Boehringer Mannheim Corporation) for 30 min at 37°C, acetylated with acetic anhydride, dehydrated in chloroform, air dried, and used immediately for hybridization. Hybridization solution was added to slides, then slides were coverslipped and incubated overnight in a humid chamber at 53.5°C. Slides were washed in increasing stringency of SSC, treated with RNase A 20 µg/ml (Boehringer Mannheim Corporation) for 30 min at 37°C, and subjected to additional washes in SSC.

At this point all slides for quantification were subjected to paired helical filament tau immunohistochemistry with mAb 69 obtained from Dr. S.H. Yen (Emory University, Atlanta, GA). Slides were incubated in blocking solution (1% horse serum, 0.5% BSA in PBS) for 3 h followed by incubation in 1:20 dilution of mAb 69 overnight at 4°C. Following washes in blocking buffer, a 1:1,000 dilution of biotinylated horse anti-mouse antibody was applied in blocking buffer for 1.5 h. Slides were washed in plain PBS then incubated in streptavidin-HRP (1:1,000) for 1 h. Immunolabeling was visualized with DAB.

Slides were dehydrated in alcohols containing ammonium acetate and exposed to film (Hyperfilm MP, Amersham Life Science Inc.) for 1 to 4 days. Slides were then dipped in undiluted NTB-2 emulsion (Kodak, Rochester, NY) and exposed for 10 to 15 days at 4°C with desiccant. Emulsion was developed in fresh D-19 (Kodak) and fixed in Kodak fixer. Slides were thoroughly washed, optionally counter-stained with Mayer’s hematoxylin, dehydrated in alcohols, cleared in xylene, and coverslipped in Cytoseal.

Quantitative Analysis of COX-1 Message Levels

Grain counting was carried out in a blind fashion on slides with short exposure (10 days). AD and control cases were matched by age, postmortem delay, and sex if possible. Paired sections from control and AD cases were mounted with care taken to position hippocampi of both cases at the same level on the slide. Emulsion grains were counted over 25 individual CA3 hippocampal neurons using a computer-assisted method (Micro Imaging Device, MCID, Toronto, CA) with a 40× objective. We have previously confirmed these methods by manual grain counting (27). Only cells with nuclei in the plane of section were included in counts. Background DAB staining was used to estimate area of individual neurons. Grain number and area were determined for each neuron, and a mean grain density for the population of 25 neurons within that section was derived. Statistical analyses were performed using paired Student’s t-test.

Quantitative Analysis of COX-1 Immunopositive Microglia

Fusiform gyrus of 9 AD cases and 9 control cases was examined. IHC using TL antibody and DAB substrate was conducted as described above, then sections were counter-stained with 0.25% cresyl violet. Using a grid reticle which marked out an area of 0.059 mm2, TL immunoreactive cells were counted in a blind fashion using a 20× objective. In the same fields we also quantified the number of neurons and the number of remaining small nuclei, assumed to primarily be glial cells. Neurons were distinguished from non-neuronal cells by nuclear size, shape, and presence of a nucleolus. Cells were counted in cortical layers I, II, and III and in underlying white matter. Three fields from different parts of the section were counted in each layer to generate mean values for each case. Comparisons between control and AD cases in each layer were analyzed using unpaired Student’s t-test.

RESULTS

COX-1 mRNA in CA3 and CA4 Hippocampal Neurons

In situ hybridization of human postmortem tissue with a full-length COX-1 antisense probe and a 353 bp specific COX-1 antisense probe showed abundant COX-1 mRNA expression in CA3 and CA4 hippocampal neurons and a few scattered CA2 neurons, but virtually no expression in CA1 neurons (Fig. 1A, B). On film exposures very low levels of COX-1 message were observed in the dentate gyrus. Low levels of COX-1 mRNA were also present in some cortical neurons (Fig. 2A). No specific signal was obtained with COX-1 sense probe (data not shown).

COX-1 Immunoreactivity in Neurons and Glia

We also examined cyclooxygenase-1 expression at the protein level. On western blots, the anti-COX-1 antibody from Oxford (PG-16) was specific for human COX-1 (Fig. 3). Kaplan et al (28) previously found that anti-human COX-2 antibody from Transduction Laboratories crossreacts with COX-1. We further verified that the TL antibody recognized both rat and human COX-1 and COX-2 on western blots (Fig. 3).

Immunohistochemistry with both TL and PG-16 antibodies revealed COX immunoreactivity (IR) in CA3 and CA4 neurons of human hippocampus (Fig. 4A–E). A demarcation was seen at the CA2–CA3 border, with only a

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few scattered CA2 neurons expressing COX-1 protein (Fig. 4C). No staining was observed in dentate gyrus granular neurons. In hippocampal neurons COX-1 IR was localized to distal and proximal dendrites and the cell body (Fig. 4C–E). We also observed a darker staining rim around the nucleus (Fig. 4E). COX-1 immunoreactivity was present in the cell bodies of granular neurons in neocortical layer IV as well as occasional pyramidal neurons in layers III and V (Fig. 2B, C). The pattern of COX-1 immunoreactivity in neurons was in complete correspondence with the results of in situ hybridization.

In addition to neuronal staining, TL and PG-16 antibodies both recognized small process-bearing cells dispersed throughout gray and white matter of control and AD tissue (Fig. 4E, F). These immunoreactive cells occasionally formed ring-like clusters in AD brain (Fig. 4F). These cells were smaller than neurons and did not have detectable Nissl substance or nuclear morphology consistent with neurons. COX-1 immunopositive cells were present in all brain regions examined: hippocampus, basal ganglia, and temporal and parietal neocortex. These cells were similar in appearance in all brain regions, except that they were more uniformly aligned along white matter tracks. This morphology and distribution is characteristic of glial cells.

Microglia Are Immunoreactive for COX-1

To identify the identity of the COX-1 immunoreactive glia, we carried out double IHC with specific glial markers. Co-localization of glial fibrillary acidic protein (GFAP) and COX-1 was not observed when sections were double-labeled with anti-GFAP antibody and PG-16 antibody (data not shown) or TL antibody (Fig. 4G). Since GFAP is a specific astrocytic marker, these results suggest that COX-1 immunopositive glial cells are not astrocytes. On the other hand, COX-1 immunopositive cells double-labeled with specific microglial markers: lectin RCA-1 and anti-HLA-DR antibody LN-3 (Fig. 4H, I). These double-labeling experiments establish that non-neuronal cells immunopositive for COX-1 are microglia.

COX-1 Immunoreactive Microglia Are Present in Areas of AD Pathology

COX-1 immunostaining in neurons and microglia was observed in both control and AD tissue. There was no apparent difference in the intensity and localization of neuronal immunostaining. Individual immunopositive microglial cells were evenly distributed throughout control tissue. However COX-1 positive microglia sometimes formed clusters in AD tissue (Fig. 4F). To determine whether the observed COX-1 IR in microglia was related to the disease state, we examined 2 markers of AD in conjunction with COX-1 IHC. We carried out double IHC with COX-1 antibody and LN-3, an antibody that recognizes HLA-DR. HLA-DR is a MHC II antigen and a marker of microglial activation in AD (29–32). As seen in Figure 4I, microglia show variable intensity of LN-3 staining in AD cortical tissue. However, COX-1 antibody labels microglia without similarity regardless of the presence or the amount of LN-3 label. Only a few cells were strongly labeled with LN-3 in control tissue and there was no clear relationship between presence or intensity of LN-3 labeling and intensity of COX-1 labeling.

Microglia surrounding neuritic plaques have characteristics indicating that they are highly activated (29, 31). Double immunohistochemistry for COX-1 and Aβ revealed that COX-1 positive microglia are imbedded in the core of amyloid plaques (Fig. 4J). However, COX-1 IR in these microglia was of similar intensity to non-plaque associated cells. We conclude that COX-1 immunoreactivity does not correlate with microglial activation. Furthermore, it appears that anti-COX-1 antibodies label all or nearly all microglia in the brain.
We were not able to verify this hypothesis since specific staining techniques that label all microglia regardless of activation state and are compatible with immunohistochemistry in human tissue are problematic (29, 31, 33, 34).

Specificity of COX-1 IR

To verify that the IR observed represented COX-1, we conducted an immunoblotting study. Microglial and neuronal immunoreactivity was blocked when TL antibody was preincubated with ovine COX-1 (Fig. 5A, B; microglial immunoreactivity shown). The PG-16 (COX-1 specific) antibody absorption study yielded the same results (data not shown). Moreover, no immunopositive microglia were observed with the COX-2 specific antibody PG-27 and an anti-human COX-2 monoclonal antibody from Cayman Chemical (Ann Arbor, MI) (data not shown). Thus the immunoreactivity pattern observed with PG-16 and TL antibodies is consistent with COX-1 protein expression in neurons and microglia.

Microglial COX-1 IR Is Not a Result of Postmortem Delay or Fixation Artifact

To assure that microglial immunoreactivity was not due to the terminal condition of the patients or postmortem interval, we performed IHC using tissue obtained during temporal cortical lobectomy from 2 different patients. This biopsy tissue was fixed 10 to 15 min following dissection. Cortical biopsy tissue from both subjects showed COX-1 immunostaining of microglia and some cortical neurons that looked identical to the postmortem tissue (Fig. 5C). We used perfused primate tissue to verify that COX-1 immunoreactivity is not an artifact of immersion fixation. IHC of macaque visual cortex showed abundant neuronal immunostaining as well as immunostaining of glia (data not shown). A very similar pattern of TL immunoreactive glia was also observed in perfused rat tissue (Fig. 5D). These data confirm that COX-1 immunoreactivity in postmortem human brain tissue is not an artifact of tissue handling, and is present in different mammalian species.

Quantitative Comparison of COX-1 Expression in AD and Control Brain

Since COX activity is potentially important in AD we compared COX-1 expression in AD and control brain. COX-1 mRNA levels in AD and control CA3 neurons were estimated by counting silver grains over individual non-tangle-bearing neurons as described in the Materials and Methods section. Sections containing the CA3 hippocampal field were not available in 5 cases. Therefore, 2 sections per case for 5 pairs of AD and control cases were used for quantification. This analysis revealed no significant difference in CA3 neuron COX-1 mRNA levels between AD and control (Fig. 6).

![Fig. 2. COX-1 expression in cortical neurons. COX-1 mRNA (A) and immunoreactivity (B) is observed in layer IV cortical neurons (arrowheads). C, COX-1 immunoreactivity is also present in occasional pyramidal neurons of neocortical layers III and V (arrowhead). Note the presence of immunoreactive glia in B and C. Scale bar = 50 μm.](http://jnen.oxfordjournals.org/)

We also quantified the density of COX-1 positive microglia as well as the density of neuronal and non-neuronal nuclei in Nissl stained sections of 9 AD and 9 control cases in which fusiform cortex was available. In AD cases there was a significant increase of 38% and 31%
in the density of COX-1 labeled microglia in cortical layers II (p < 0.02) and III (p < 0.02), respectively. An increase in density of immunopositive glia was insignificant in cortical layer I and white matter (Fig. 7A).

In AD brain an increase in the number of COX-1 positive cells per field may be due to neuronal loss and shrinkage of the neuropil, which occurs in AD (35), and glial proliferation. To account for this we normalized the density of COX-1 positive cells to the total density of non-neuronal cells. As a result there was no significant difference in the proportion of cells expressing COX-1 in AD and control in all layers examined (Fig. 7B). Thus, the same proportion of glial cells is immunoreactive for COX-1 in control and AD. However, in AD there is an absolute increase in the density of COX-1 expressing microglia in layers II and III.

DISCUSSION

Precise localization of COX-1 in the mammalian brain is not known and has been hampered by cross-reacting antibodies (20, 36, 37). In this study we show COX-1 mRNA and protein in human CA3 and CA4 hippocampal and some cortical neurons. COX-1 immunoreactive neurons were previously reported in ovine and monkey brain (38, 39). In both studies antisera was raised against purified COX-1 but isoform specificity of antisera was not established. Trabokura et al (38) observed strong COX-1 IR in virtually all monkey cortical neurons; however, antibody preabsorption with COX-1 did not completely abolish immunoreactivity. Breder et al (39) reported that COX-1 staining was stronger in CA1 than CA3–CA4 hippocampal neurons; they also observed COX-1 immunoreactivity in dentate gyrus. These observations do not agree with our results and may be due to problems with antibody specificity or differences between species. Kawasaki et al (40) detected COX-1 in rat brain on Northern blots. Li et al (41) observed COX-1 mRNA in various rat brain neuronal populations using a human COX-1 probe. Norton et al (42) studied fetal ovine brain and showed strong COX-1 mRNA signal in different cortical and subcortical neuronal groups including dentate gyrus. We observed only very low levels of COX-1 message in human dentate gyrus neurons. This discrepancy can be attributed to differences in species and developmental stage. COX-2 expression in brain has been studied in more detail, and has been shown in all hippocampal CA fields, DG, subiculum, as well as certain cortical lamina (43–48). Comparison of COX-1 and 2 distribution

Fig. 4. Characterization of COX-1 immunoreactivity. In human brain COX-1 immunoreactivity localizes to CA3 and CA4 zones of hippocampus as seen with PG-16 (A, E) and TL (B, C, D) antibodies. Note the demarcation in staining between CA3 and CA2 fields (C). COX-1 immunoreactivity in hippocampal neurons is localized to cell bodies and dendrites (D); stronger immunoreactivity is observed in the perinuclear region (E, large arrow). E, PG-16 (anti-COX-1) antibody immunopositive glia, dispersed throughout gray matter and white matter, are much smaller (small arrows) than neurons (large arrow). F is a high power view of COX-1 IR microglia in AD tissue using the TL antibody. Note the ring-like cluster of microglia in the center and evenly distributed microglia in the remainder of the section. Panels G through J depict double immunohistochemistry of human temporal neocortex. Although identical results were obtained with PG-16 antibody, TL immunohistochemistry is presented in these panels since this antibody provides a more distinct signal. In G, absence of double labeling with anti-GFAP antibody (brown; arrows) shows that small COX-1 immunopositive cells (blue-gray) are not astrocytes. In H, TL antibody labeled cells (blue-gray) have processes that label with the lectin RCA-1 (brown; arrows) demonstrating that COX-1 immunopositive cells are microglia. In I, intensity of COX-1 immunolabel (blue-gray) does not vary with intensity of LN-3, a specific marker of microglial activation (red). In AD cortex (J) COX-1 immunopositive microglia (brown) are embedded in the core of amyloid plaques (10D5 antibody, blue-gray). Scale bar = 800 μm in A and B, 200 μm in C, 100 μm in D, 50 μm in E, F, I and J, 33 μm in G, and 20 μm in H. Cresyl violet counter-stain was used in B and C.
Fig. 5. Specificity of TL antibody in immunohistochemical applications. A, Human cortex was immunolabeled with TL antibody using standard techniques and a DAB substrate. In panel B the primary antibody was preabsorbed with ovine COX-1. Note the clear and specific staining of glia in panel A, versus the absence of specific signal in panel B. Human cortical biopsy (C) and rat basal ganglia (D) show the same pattern of COX-1 immunoreactive glia. Scale bar = 50 μm.

Fig. 6. COX-1 mRNA levels in AD and control. Bars represent the density of COX-1 emulsion grains (mean ± SEM) over individual CA3 hippocampal neurons in AD and control cases (n = 5). These values are not significantly different from one another using a paired Student's t-test.

suggests that some hippocampal neurons express both, while others express only one COX isoform.

COX-1 is induced in differentiated PC12 cells (28), but its regulation and function in cortical neurons has not been studied. We report that the intracellular distribution of COX-1 in human hippocampal neurons is very similar to that of COX-2, with protein found both in the cell body and in dendrites. Thus the functional role of the 2 proteins in brain may be similar. One hypothesis is that COX is involved in synaptic signaling. In support of this hypothesis, prostaglandin production and cortical COX-2 expression are induced by electrical activity during normal behavior and seizures (45, 46, 49–52). Moreover, Kaufmann et al (48) reported that COX-2 is expressed in dendritic spines, and a series of studies indicates that COX and prostanoids are involved in NMDA receptor-mediated induction of c-fos (53–55).

Studies using selective inhibitors suggest that COX-2 contributes to neuronal vulnerability in models of focal and global brain ischemia (21, 22). Although the mechanism is not understood, cyclooxygenases produce free
neurons of the CA1 zone and subiculum (35, 57). Furthermore, our data indicate that COX-1 mRNA levels in CA3 neurons remain unchanged in AD. This observation is consistent with a recent study by Pasinetti and Aisen (58). Taken together, these data suggest that intracellular COX-1 does not make hippocampal neurons more vulnerable to the pathological processes active in AD.

The capacity of microglia to produce prostaglandins is well established (59). COX-2 is regulated in microglia in vitro; however, there is a lack of evidence for microglial COX-2 expression in vivo (20). Similarly, COX-1 expression has been previously shown in cultured microglia (60, 61), but in vivo data are scarce. Tsubokura et al (38) observed COX-1 IR glia in all regions of monkey brain, but did not determine the identity of those cells. Miettinen et al (62) observed microglia-like immunoreactive cells in rat brain using the same anti-COX antibody from Transduction Laboratories used in our analysis. In the present study we show COX-1 immunoreactivity in human microglia. We were unable to show COX-1 expression in microglia at the message level. This is most likely due to the small size of microglia and low quantity of message expressed.

Microglia are important players in CNS inflammation, immune and degenerative processes (59, 63, 64). In AD brain reactive phagocytic microglia are associated with amyloid plaques (29, 65, 66). There is accumulating evidence that microglial activation and migration to amyloid plaques precedes neuronal and astrocytic pathology and may represent one of the earliest events in neuritic plaque formation (67–70). Although there is some indication that microglia do not initiate plaque formation (66, 69, 71), cultured microglia synthesize amyloid precursor protein (APP) and metabolize APP in vitro in a way that favors Aβ production (72–75). Aβ can activate microglia in vitro (76, 77) and in vivo (78, 79). Furthermore, microglia associated with plaques express high levels of IL-1, a known proinflammatory cytokine, which upregulates expression and processing of APP, promotes microglial proliferation, induces IL-6, TNFα, IL-1, and has trophic effect on astrocytes (80). Finally, transgenic mice expressing mutated human APP show a significant increase in microglial density around amyloid deposits (81). Thus, microglia are believed to contribute to AD pathogenesis by playing an active role in plaque maturation as well as induction and maintenance of inflammatory responses to Aβ.

Netland et al (82) showed that rats receiving intraventricular infusions of Aβ peptide exhibited pronounced microglial activation. This response was significantly attenuated in animals treated with the nonselective COX inhibitor indomethacin. However, additional quantitative studies and verification of specificity of COX-6 staining are needed to confirm these findings. Another recent study showed that nondemented elderly arthritic patients
with documented use of NSAIDs had 3 times fewer activated microglia than control subjects lacking a history of NSAID use (83). These studies suggest that inhibition of COX by NSAIDs can reduce microglial activation in response to Aβ.

In AD brain we observed increased numbers of COX-1 expressing cells per unit area in certain cortical layers and clusters of COX-1 expressing microglia around amyloid plaques. Our findings are consistent with a modest increase of COX-1 protein reported in AD temporal cortex (84). The numbers we observed are comparable to those reported by Carpenter et al (32) using the LN-3 antibody that stains HLA-DR. However, in AD tissue we did not detect as profound an increase in COX-1 immunostained microglia as they reported using LN-3 as a microglial marker. One explanation may be that COX-1 immunoreactivity is detected in all microglia; whereas, MHC class II staining is dependent on activation state. Indeed, although immunohistochemical staining intensity is at best a semiquantitative measure, we found no evidence correlating intensity of microglial COX-1 immunostaining with activation state indicated by staining for HLA-DR. Nevertheless, the greater density of COX-1 expressing microglia in AD, particularly in the vicinity of plaques, suggests that prostanoid production is increased in AD. Prostanoid production may be further augmented by increased availability of arachidonic acid substrate, produced by elevated expression of cytoplasmic phospholipase A2, in AD (85, 86). PGE measurements in postmortem tissue are problematic and so far inconclusive (87, 88). Because thromboxanes are relatively specific to microglia (89), quantification of thromboxane levels in CSF or postmortem brain may provide a useful measure of prostaglandin production and microglial activation in AD.

Since currently available NSAIDs inhibit both isoforms of COX, the contribution of each isoform to AD progression remains an open question. We have identified COX-1 as a potential substrate for NSAID action in human brain. In brain COX-1 is expressed in 2 different cell populations: neurons and microglia. Future studies should consider that NSAIDs modify COX-1 activity in both cell types, and expression of COX-1 in either or both of these cell populations may contribute to the progression of AD. Ongoing clinical trials using selective COX-2 inhibitors will help to sort out the contributions of each isoform.

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REFERENCES


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64. Kreutzberg GW. Microglia: A sensor for pathological events in the CNS. Trends Neurosci 1986;19:312–18

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