Neuronal Cyclooxygenase 2 Expression in the Hippocampal Formation as a Function of the Clinical Progression of Alzheimer Disease

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Background: Prior studies have shown that cyclooxygenase 2 (COX-2), an enzyme involved in inflammatory mechanisms and neuronal activities, is up-regulated in the brain with Alzheimer disease (AD) and may represent a therapeutic target for anti-inflammatory treatments.

Objective: To explore COX-2 expression in the brain as a function of clinical progression of early AD.

Design and Main Outcome Measures: Using semi-quantitative immunocytochemistry, we analyzed COX-2 protein content in the hippocampal formation in 54 post-mortem brain specimens from patients with normal or impaired cognitive status.

Setting and Patients: Postmortem study of nursing home residents.

Results: The immunointensity of COX-2 signal in the CA3 and CA2 but not CA1 subdivisions of the pyramidal layers of the hippocampal formation of the AD brain increased as the disease progressed from questionable to mild clinical dementia as assessed by Clinical Dementia Rating. COX-2 signal was increased in all 3 regions examined among cases characterized by severe dementia.

Conclusion: Neuronal COX-2 content in subsets of hippocampal pyramidal neurons may be an indicator of progression of dementia in early AD.

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A LARGE NUMBER of epidemiologic studies have indicated that the use of non-steroidal anti-inflammatory drugs (NSAIDs) may prevent or delay the clinical features of Alzheimer disease (AD).1-3 The pharmacologic activity of NSAIDs is generally attributed to inhibition of cyclooxygenase (COX), a rate-limiting enzyme in the production of prostaglandins. Two distinct COX isoforms have been characterized: a constitutive form, COX-1, and a mitogen-inducible form, COX-2.4 Characterization of COX expression in the brain may be important to understanding the potential therapeutic effect of NSAIDs and to devising optimal treatment regimens.

We5-8 and others9-14 found that the expression of neuronal but not glial COX-2 is elevated in the AD brain, where it may be involved in neuritic plaque (NP)6 and neurofibrillary tangle (NFT) pathologic conditions.7 The role of COX-2 in AD neurodegeneration is incompletely understood but may include potentiation of β-amyloid (Aβ)6 and glutamate6 neurotoxicity. In the present study, we further explored COX-2 expression as a function of the clinical progression of AD dementia.

RESULTS

CASES

Age, postmortem interval, neuropathologic findings, and other information are shown in the Table. There was no significant difference in mean age or mean postmortem interval (ANOVA; P = .40 and P = .82, respectively) among the CDR groups. Within each CDR group, the average hippocampal neuronal COX-2 immunostaining intensity did not differ among cases with or without a previous history of NSAID or steroid use (not shown). Cases of patients with a history of inflammatory conditions (eg, sepsis) were excluded from the analysis.

COX-2 CONTENT IS ELEVATED AS A FUNCTION OF CLINICAL DEMENTIA

The characterization of immunostained hippocampal pyramidal cells as neurons...
**MATERIALS AND METHODS**

**POSTMORTEM HUMAN BRAIN**

Human postmortem brain specimens from cases with normal or impaired cognitive status were obtained from the Alzheimer’s Disease Brain Bank of the Mount Sinai School of Medicine (MSSM). A multistep approach based on cognitive and functional status during the last 6 months of life was applied to the assignment of Clinical Dementia Rating (CDR) scores as previously reported. Subjects were divided into groups on the basis of their CDR scores as follows: 0, none; 0.5, questionable dementia; 1, mild dementia; 2, moderate dementia; and 4 to 5, severe or very severe dementia. The extent of NFTs and NP neuropathologic findings was assessed in accord with the Consortium to Establish a Registry for Alzheimer’s Disease (CERAD) neuropathologic battery. Multiple (5 in general) high-power (×200, 0.5-mm²) fields were examined in each histological slide. The density of NFTs and NPs was rated on a 4-point CERAD scale: 0, none; 1, sparse; 3, moderate; and 5, frequent and severe. The investigators were blind to the diagnosis of each case until all quantitative analysis was completed and values were assigned to each specimen. All assessment were approved by the MSSM Institutional Review Board. Autopsies were performed after receiving consent from each subject’s legal next of kin.

**IMMUNOCYTOCHEMISTRY**

Paraffin-embedded brain tissue sections encompassing the ventral hippocampal formation (10 μm) were deparaffinized, hydrated in descending concentrations of ethanol, and reacted with either an anti–human COX-2 (Cayman Chemical Co, Ann Arbor, Mich; 1:300 dilution) or an anti–human COX-2 antiserum from BD Transduction Laboratories (Burlington, Ky; 1:250 dilution) and reacted with either an anti–human COX-2 (BD Transduction Laboratories) or a monoclonal antibody to NSE (Dako Corp, Carpinteria, Calif; 1:200 dilution). In this study, the anti–human COX-2 antibody used in our study reacts specifically with purified human recombinant COX-2 but not with human recombinant COX-1 peptide. Each immunocytochemistry experiment included 3 to 5 sets of hippocampal tissue sections from different cases across all CDRs (CDR 0 to CDR 5). All specimens were subjected to identical primary and secondary biotinylated antibody treatment (from identical prediluted stocks) and diaminobenzidine staining as previously described. The Vectastain ABC kit (Vector, Burlingame, Calif) was used in subsequent steps to complete the diaminobenzidine staining as previously described. We previously showed that the anti–human COX-2 antiserum used in this study reacts specifically with purified human recombinant COX-2 but not with human recombinant COX-1 peptide. Each immunocytochemistry experiment included 3 to 5 sets of hippocampal tissue sections from different cases across all CDRs (CDR 0 to CDR 5). All specimens were subjected to identical primary and secondary biotinylated antibody treatment (from identical prediluted stocks) and diaminobenzidine staining. Control tissue sections incubated in the absence of primary antibody gave negative staining.

The immunostaining densities of COX-2 or NSE over pyramidal layers of the hippocampal formation were digitized with a high-resolution charge-coupled-device camera (Sony, Tokyo, Japan) and quantified using Bioquant computer-assisted densitometry (Bioquant, Nashville, Tenn) as previously described. Camera aperture and focus were adjusted to provide an optimal image. The overall illumination was also adjusted so that the distribution of relative gray values, ie, number of pixels in the image as a function of gray value (0-255), fell within the limits of the system, typically within 30 to 220 gray value units, avoiding a floor or ceiling effect. Once established, the setting remained constant for all the images acquired for all the immunocytochemistry experiments. Therefore, when all the parameters were fixed, only tissue staining intensities influenced the measured gray value. Images, acquired as described, were digitized and stored for later analysis using an IBM-compatible computer.

Average gray value density measurements from individual hippocampal neurons, which reflected immunostaining intensity, were made on digitized images by delimiting the cellular area of interest free hand, using predetermined criteria to define the region of interest. The intensity of the cellular COX-2 and NSE immunostaining per cell was quantified from approximately 6 to 8 frames per section encompassing the hippocampal pyramidal layers; about 6 to 10 neurons per frame were randomly quantified. The technician who performed these measurements had no knowledge of the subject’s CDR. To normalize any unevenness in lighting across the field of view, background gray values were determined over the white matter area (cortical white matter that gave no cellular staining) of each individual tissue section and automatically subtracted from the gray values over hippocampal pyramidal neurons of the corresponding tissue section. All data were expressed as the percentage of the mean value for CDR 0 cases.

**QUANTIFICATION OF Aβ1-42 CONTENT**

Cortical Aβ1-42 was extracted and quantified as previously described. Briefly, frozen tissue samples (100 mg) were homogenized in buffer containing 70% formic acid and 100 mmol/L betaine, and the soluble Aβ1-42 was quantified by enzyme-linked immunosorbent assay (ELISA) using synthetic Aβ1-42 (US Peptides, Fullerton, Calif) as a standard. Microwell plates were coated with 2 mg/mL monoclonal antibody 4G8 (Senetek, Maryland Heights, Mo), which recognizes an epitope between residues 17 and 20 of Aβ. Unoccupied binding sites on the plates were blocked by incubation with casein. Samples and standards were applied in quadruplicate and incubated for 48 hours at 4°C. Following the Aβ1-42 capture phase, the plates were reacted with an Aβ1-42 C-terminal–specific antibody followed by incubation with a reporter antibody (alkaline phosphatase–conjugated anti–rabbit IgG, γ-chain–specific; JBL Scientific, San Luis Obispo, Calif). The assay was developed using an alkaline phosphatase substrate (Attophos; JBL Scientific), yielding a fluorescent product, and analyzed with a 96-well fluorescence reader (CytoFluor; Millipore, Bedford, Mass). All samples were analyzed in the linear range of the ELISA.

**STATISTICS**

Statistical analysis was performed using the Prism software package (GraphPad Software, Inc, San Diego, Calif). Analysis of variance (ANOVA) was used to evaluate differences in mean values among 3 or more groups, and the Dunnett t test was used to test the significance of differences in mean immunointensities. One-tailed or 2-tailed tests were used as indicated. The Welch correction for unequal variance was applied when appropriate. Correlation analysis between 2 variables was done using the Pearson parametric method followed by 2-way analysis of P value.
Figure 1. Elevated cyclooxygenase 2 (COX-2) immunostaining in pyramidal neurons of the hippocampal formation in a cognitive normal control brain and in a brain with moderate dementia. Representative micrographs of COX-2 immunostaining among neurons in the CA3 subdivision of the hippocampal pyramidal layer. A, Cognitive normal control brain with a Clinical Dementia Rating score of 0. B, Moderate dementia brain with a Clinical Dementia Rating score of 2. Arrows point to COX-2–immunolabeled cells. Scale bar equals 20 µm.

Figure 2. Cyclooxygenase 2 (COX-2) immunostaining is elevated in the CA2 through CA3 pyramidal neurons of the hippocampal formation of the brain with Alzheimer disease. Quantification of COX-2 signals in neuronal cells of the CA3 (A), CA2 (B), and CA1 (C) subdivisions of the hippocampal pyramidal layer are shown as a function of the Clinical Dementia Rating (CDR) score. Bar graphs represent mean±SEM of neuronal COX-2 immunostaining intensity as a percentage of CDR 0 from approximately 6 to 8 frames encompassing the hippocampal neuronal layers (about 6 to 10 neurons per frame). One-tailed Dunnett * test vs CDR 0: asterisk indicates P<.05; dagger, P<.005; and double dagger, P<.001. Inset, Anatomical map depicting the 3 subdivisions within the pyramidal layer of the hippocampal formation.

Characteristics of Study Subjects*

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<th>CDR Score</th>
<th>No. of Subjects</th>
<th>Mean ± SEM Postmortem Interval, h</th>
<th>Mean ± SEM Age, y</th>
<th>Female, %</th>
<th>Median CERAD Plaque Rating</th>
<th>Median CERAD Tangle Rating</th>
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<td>80 ± 3</td>
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<td>13 ± 6.5</td>
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* CDR indicates Clinical Dementia Rating; CERAD, Consortium to Establish a Registry for Alzheimer’s Disease.
or glia was based on location within the pyramidal layers of the hippocampal formation and cell morphologic structure and size. Neuronal COX-2 immunostaining in pyramidal neurons of the hippocampal formation was compartmentalized to the perikarya and processes (Figure 1A-B). There was an overall elevation in COX-2 immunostaining in pyramidal neurons of the CA3, CA2, and CA1 subdivisions of the hippocampal formation as a function of the clinical progression of AD dementia by CDR (ANOVA; NP, P < .05, NFT, P < .005) (Figure 2A-B). No elevation of COX-2 immunostaining as a function of NP and NFT neuropathologic findings was found in the CA2 subdivision (ANOVA; NP, P = .10; NFT, P = .06) (Figure 4C-D) or CA1 (ANOVA; NP, P = .91; NFT, P = .07) (Figure 4E-F) subdivisions.

Relative to cases characterized by no NP pathologic findings (CERAD 0), definitive elevation of COX-2 immunostaining in the neurons of the CA3 (Figure 4A) subdivision was found in cases characterized by moderate NP pathologic findings (CERAD 3) (P < .01). Elevated COX-2 immunostaining in the CA3 subdivision (Figure 4B) was also found in cases characterized by severe NFT pathologic findings (CERAD 5) (P < .005) (Figure 4B).

The goal of this study was to explore COX-2 expression as a function of the early stages of clinical AD. We found that COX-2 is elevated in subsets of neurons of the hippocampal formation during early dementia. In particular, the intensity of COX-2 immunostaining in the neurons of the CA2 and CA3 but not CA1 subdivisions of the pyramidal neuron layer of the hippocampal formation rose as the disease progressed from questionable AD dementia (CDR 0.5) to mild (CDR 1) clinical stages. The changes in COX-2 immunostaining in the CA2 and...
CA3 subdivisions of the hippocampal formation of cases characterized by mild dementia were rather specific, since no detectable increase of NSE immunostaining was found. Among individual cases, COX-2 signal in these neuronal layers correlated with total cortical Ab1-42 content. When the cases examined were stratified by neuropathology ratings, we found that the neuronal COX-2 immunostaining was selectively elevated in the CA3 subdivision of the pyramidal neuron layer of cases characterized by moderate and severe NP and NFT neuropathologic findings, respectively, relative to cases characterized by CERAD 0. No overall elevation of COX-2 immunostaining was found in the CA2 and CA1 subdivisions of the pyramidal layer as a function of NP and NFT neuropathologic findings. COX-2 was elevated (>50%) in the CA2 subdivision of cases characterized by moderate NP neuropathologic findings; however, we point out that this finding must be viewed with some caution in light of multiple comparisons. The data provide a rational basis for targeting COX-2 activity in therapeutic trials aimed at the prevention and treatment of early AD.

Several pharmaceutical company- and government-sponsored trials are currently investigating the therapeutic potential of NSAIDs with regard to AD. The results of 2 small pilot studies of NSAIDs have been published; one suggested a neuroprotective effect with indomethacin treatment, whereas the other reported equivocal results with diclofenac. Current randomized, placebo-controlled trials testing the efficacy of NSAIDs and other agents select subjects based on clinical criteria such as CDR score and cognitive test results.
The optimal design of such studies should consider the expression of the presumed molecular target of the therapeutic intervention at different clinical stages of disease. The presumed mechanism of the possible beneficial effect of NSAIDs in AD involves COX inhibition. Thus, elucidation of the role of COX-2 in mechanisms of neural degeneration in various clinical stages of dementia will certainly aid the rational design of such trials.

We found that COX-2 immunostaining was preferentially increased in the CA3 and, to a lesser extent, in the CA2 subdivision of the hippocampal pyramidal layer of cases characterized by moderate and severe NP and NFT pathologic findings. However, there was no increase in COX-2 immunostaining with neuropathologic progression in the CA1 subdivision, which is also highly vulnerable to NFT neuropathologic findings. This result suggests that COX-2 regulation may involve qualitatively different mechanisms in specific subsets of neurons of the hippocampal formation, consistent with previous findings.

In conclusion, the present study provides evidence for involvement of COX-2 in hippocampal neuronal pathologic conditions during mild AD dementia. Elevated expression of COX-2 in subdivisions of the hippocampal formation is correlated with progression of clinical disease stage. Although controlled trials of therapeutic interventions directed at COX-2 inhibition may thus be appropriate at any stage of disease, these data suggest that trials that include subjects at early stages may be particularly promising.

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REFERENCES