Research paper

Cyclooxygenase-1 null mice show reduced neuroinflammation in response to β-amyloid

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Abstract: Several independent epidemiological studies indicate that chronic use of non-steroidal anti-inflammatory drugs can reduce the risk of developing Alzheimer's disease (AD), supporting the inflammatory cascade hypothesis. Although the first clinical trial with indomethacin, a preferential cyclooxygenase (COX)-1 inhibitor, showed beneficial effects, subsequent large clinical trials, mostly using COX-2 inhibitors, failed to show any beneficial effect in AD patients with mild to severe cognitive impairment. These combined data suggest that either an early treatment is crucial to stop the mechanisms underlying the disease before the onset of the symptoms, or that preferential COX-1 inhibition, rather than COX-2, is beneficial. Therefore, a full understanding of the physiological, pathological, and/or neuroprotective role of COX isoforms may help to develop better therapeutic strategies for the prevention or treatment of AD. In this study, we examined the effect of COX-1 genetic deletion on the inflammatory response and neurodegeneration induced by β-amyloid. β-amyloid ($A\beta_{1-42}$) was centrally injected in the lateral ventricle of *COX-1*-deficient (*COX-1*$^{-/-}$) and their respective wild-type (WT) mice. In *COX-1*$^{-/-}$ mice, $A\beta_{1-42}$-induced inflammatory response and neuronal damage were attenuated compared to WT mice, as shown by Fluoro-Jade B and nitrotyrosine staining. These results indicate that inhibition of COX-1 activity may be valid therapeutic strategy to reduce brain inflammatory response and neurodegeneration.

INTRODUCTION

Alzheimer's disease (AD) is an aging-related progressive neurodegenerative disease, characterized by massive neuronal and synaptic loss, accompanied by neuropathological changes, such as neurofibrillary tangles and senile plaques, in the hippocampus, neocortex, and subcortical structures [1]. The senile plaques are primarily composed of amyloid beta peptide (Aβ), which is a 40–42 amino acid peptide fragment of the amyloid protein precursor. However, the mechanism by which Aβ causes neuronal injury and cognitive impairment is unclear. AD is also thought to have a local, non-immune mediated neuroinflammatory component with clusters of activated microglia, increased inflammatory proteins (complement factors, acute-phase protein, pro-inflammatory cytokines) [2-4], and increased COX-1-expressing microglia surrounding amyloid plaques [2]. Changes in COX-2 expression in AD are discrepant and seem to depend on the stage of the disease, with an upregulation of COX-2 in early AD, and a downregulation in advanced AD stages, which also correlate with PGE_2 levels in the CSF, which are increased in probable AD patients and decrease with the progression of the disease [5, 6]. Several independent epidemiological studies have shown that early use of non steroidal anti-inflammatory drugs (NSAIDs), which inhibit COX activity, significantly reduces the risk of developing AD later in life suggesting that inflammation is critical for the

progression of the disease [7-13]. However, although a 6-month, double-blinded, placebo-controlled study with indomethacin, a preferential COX-1 inhibitor, appeared to protect AD patients from cognitive decline [14], subsequent large-scale randomized clinical trials, mostly with selective COX-2 inhibitors, did not show any beneficial effects in AD patients with mild to severe symptoms [15-18]. Supporting these clinical data, indomethacin, but not the COX-2 selective nimesulide, significantly reduced levels of Aβ in the hippocampus and cortex of transgenic mouse models of AD [19]. While the clinical data seem to rule out a protective effect of selective COX-2 inhibition in AD, it is still unclear whether COX-2 inhibitors can improve the pathology in animal models of AD. For instance, COX-2 inhibition blocks Aβ-mediated suppression of long-term potentiation and memory function, independently of reductions in $A\beta_{1-42}$ or in inflammation [20]. However, the selective COX-2 inhibitor celecoxib has been shown to increase Aβ levels [21, 22], and in a model of acute inflammation, both genetic deletion and pharmacological inhibition of COX-2 worsen the neuroinflammatory response to lipopolysaccharide (LPS) [23]. These combined data suggest that either NSAIDs have rather a preventive than a therapeutic effect or that preferential COX-1 inhibition is a better therapeutic approach than selective targeting COX-2, or that the beneficial effects are due to COX-independent effects of NSAIDs. In particular, ibuprofen, flurbiprofen, and diclofenac have been shown to reduce serum $A\beta_{1-42}$ levels, a major component of senile plaques in AD [24-28]. However, a recent report from a pooled dataset from six prospective studies indicated that NSAIDs use reduced the risk of AD without any apparent advantage for the subset of NSAIDs shown to selectively lowering $A\beta_{1-42}$ [29]. While COX-1 and COX-2 are both differentially expressed in different stages of AD pathology, their specific roles in the pathogenesis of AD is unclear. Therefore, a full understanding of the physiological, pathological, and/or neuroprotective role of COX isoforms may help to develop better therapeutic strategies for the prevention or treatment of AD.

Partial reproduction of AD neuropathology and cognitive deficits has been achieved with pharmacological and genetic approaches. Most injection models use synthetic peptide $A\beta_{1-40}$ or $A\beta_{1-42}$, which are analogous to peptides found in neuritic plaques in AD patients [30]. Mice with a null mutation of COX gene have been a useful tool for investigating the role of each COX isoform in both physiological and pathological conditions in the CNS by overcoming the complexity of dosing paradigm, duration of treatment, and possible unspecific inhibition of both COX isoform [31]. In this study, we assessed the effect of intracerebroventricular (i.c.v.) injection of $A\beta_{1-42}$ on acute neuroinflammatory response in *COX-1*-deficient (*COX-1*$^{-/-}$) mice and their respective wild-type mice (WT) controls. We showed that *COX-1*$^{-/-}$ mice are more resistant than WT mice to $A\beta_{1-42}$-induced neuronal death and exhibit a marked reduction in the inflammatory response.

RESULTS

The inflammatory response is reduced in *COX-1*$^{-/-}$ mice after $A\beta_{1-42}$ injection

$A\beta_{1-42}$ or the control reverse peptide $A\beta_{42-1}$ was unilaterally injected into the lateral ventricle, as reported [32-35]. Seven days later, brains were removed and coronal sections were processed for immunohistochemistry. We assessed microglial activation in the brain using IBA-1 as a microglial marker. $A\beta_{1-42}$ administration caused a robust inflammatory response within the CA1 and CA3 areas of the hippocampus of WT mice characterized primarily by the presence of activated microglia (Figure 1A, D, J). Intense IBA-1-immunoreactive microglia with enhanced staining intensity, retracted processes, perikaryal hypertrophy, and amoeboid appearance were observed in the CA3 area of hippocampus of WT mice (Figure 1G). In *COX-1*$^{-/-}$ mice, IBA-1-immunreactive microglia retained a resting morphology with specifically small cell bodies, thin, and ramified processes (Figure 1B, E, H, J). In reverse peptide $A\beta_{42-1}$-injected mice, only a few faintly IBA-1-immunoreactive microglia were observed in the hippocampus (Figure 1C, F, I, J). Staining with CD11b, another marker for microglia gave results similar to that of IBA-1 (data not shown).

We then assessed astrocytes immunoreactivity by staining the brain of WT and *COX-1*$^{-/-}$ mice with the astrocytic marker glial fibrillary acidic protein (GFAP). GFAP-immunoreactive astrocytes in response to $A\beta_{1-42}$ injection were markedly attenuated in the brain of *COX-1*$^{-/-}$ mice (Figure 2B, E, H) compared to WT mice (Figure A, D, G). These results indicate that $A\beta_{1-42}$ administration induced less severe glial cell activation in *COX-1*$^{-/-}$ mice compared to WT mice.

COX-1 deficiency leads to reduced neuronal damage following $A\beta_{1-42}$ injection

We next assessed neuronal damage in the brain using the fluorescent marker Fluoro-Jade B (FJB), which selectively labels injured neurons [36, 37]. $A\beta_{1-42}$ administration caused a significant neuronal damage, characterized by the presence of FJB-positive neurons

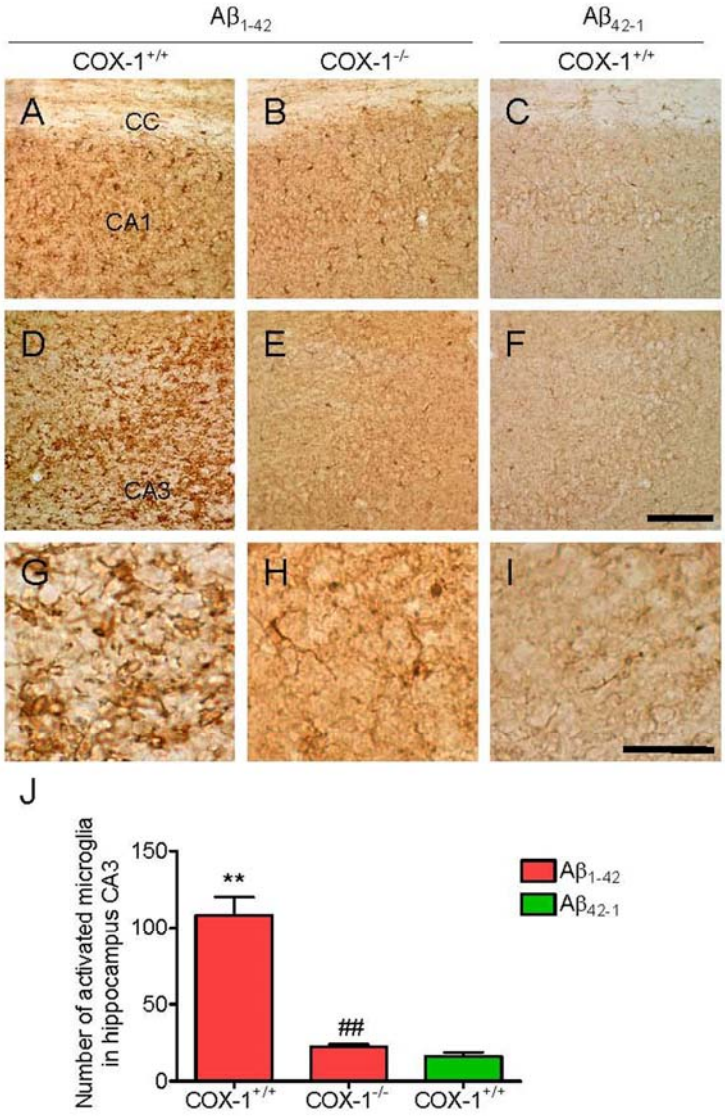

Figure 1. Increased microglial activation in the hippocampus 7 d after $A\beta_{1-42}$ administration. Representative photomicrographs of the CA1 and CA3 of the hippocampus from WT mice (**A, D**) injected with $A\beta_{1-42}$ that shows numerous activated microglia with short, less-ramified processes, perikaryal hypertrophy, and amoeboid appearance (**G**). CA1 and CA3 areas of the hippocampus from $A\beta_{1-42}$–injected *COX-1*$^{-/-}$ mice (**B, E**) show many resting microglia with ramified morphology (H). Scale bar: A-F, 100 µm; G-I, 50 µm. (**J**) Comparison of the number of activated microglia from the CA3 area. Mean ± SEM (*n* = 3-4 per group); $^{**}P < 0.01$ compared with the $A\beta_{42-1}$-injected WT mice; $^{\#\#}P < 0.01$ compared with the $A\beta_{1-42}$-injected WT mice.

within the CA3 areas of hippocampus of WT mice (Figure 3A, J). In contrast, $A\beta_{1-42}$-injected *COX-1*$^{-/-}$ mice showed few scattered FJB-positive neurons in the CA3 of hippocampus (Figure 3B, J). In same sections stained with DAPI or adjacent sections stained with cresyl violet, a similar distribution of neuronal loss and gliosis was found in the CA3 areas of hippocampus in $A\beta_{1-42}$-injected WT mice (Figure 3D, G). FJB and Nissl staining showed that hippocampal CA3 neurons in *COX-1*$^{-/-}$ mice were better preserved than in WT mice (Figure 3E, H). These results indicate that $A\beta_{1-42}$ administration induced less severe neuronal damage in *COX-1*$^{-/-}$ mice compared to WT mice.

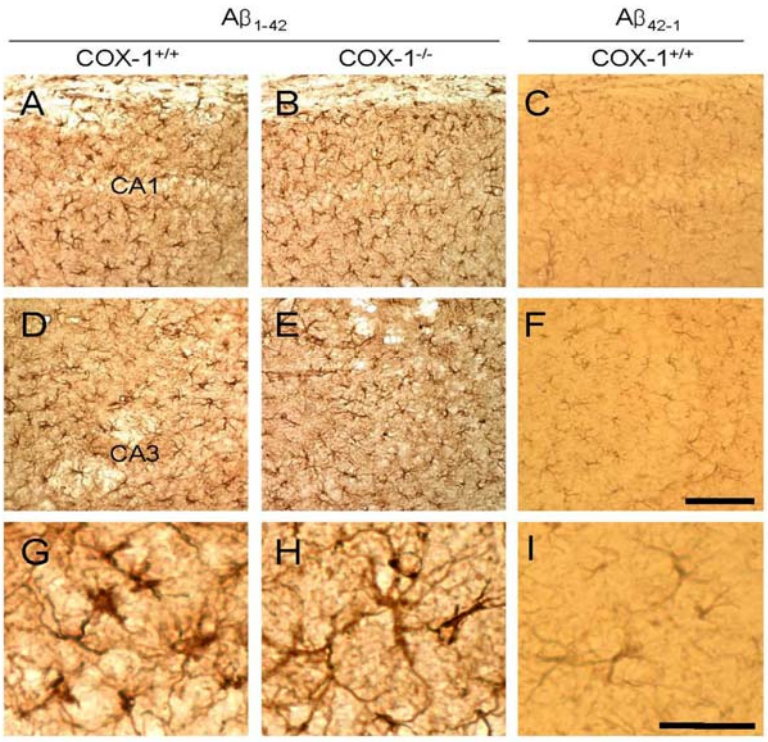

Figure 2. Increased astrocytic activation in the hippocampus 7 d after $A\beta_{1-42}$ administration. Representative photomicrographs of the CA1 and CA3 of the hippocampus from WT mice (**A, D, G**) injected with $A\beta_{1-42}$ that shows numerous robustly GFAP-immunoreactive astrocytes compared with $A\beta_{1-42}$–injected *COX-1*$^{-/-}$ mice (**B, E, H**). Scale bar: A-F, 100 µm; G-I, 50 µm.

COX-1$^{-/-}$ mice exhibit reduced oxidative damage following $A\beta_{1-42}$ administration

An important component of $A\beta_{1-42}$-induced neurotoxic process is mediated by oxidative damage [38], which can be evaluated by assessing protein carbonyls and nitrotyrosine levels [39]. To determine whether oxidative damage is involved in the process of $A\beta_{1-42}$-induced neurotoxic process, we investigated oxidized amino acid, nitrotyrosine levels using sections adjacent to those used for FJB staining. We found an increase in nitrotyrosine-immunoreactive cells in the brain of WT mice (Figure 4A, D), which was markedly attenuated in the brain of *COX-1*$^{-/-}$ mice (Figure 4B, E). These results indicate that $A\beta_{1-42}$ administration induced less severe oxidative damage in *COX-1*$^{-/-}$ mice compared to WT mice.

PG generation is reduced in $A\beta_{1-42}$-injected *COX-1*$^{-/-}$ mice

To determine the contribution of COX-1 to PG production after $A\beta_{1-42}$ injection, we measured the levels of PGE_2, $PGF_{2\alpha}$, and TXB_2 24 h after $A\beta_{1-42}$ administration. We observed significant reduction in levels of PGE_2 (Figure 5A), $PGF_{2\alpha}$ (Figure 5B), and TXB_2 (Figure 5C) in $A\beta_{1-42}$-injected *COX-1*$^{-/-}$ mice.

These results suggest that the reduced levels of PGE_2, $PGF_{2\alpha}$, and TXB_2 in *COX-1*$^{-/-}$ mice could contribute, in part, to the observed differences in glial and neuronal response to $A\beta_{1-42}$ administration.

DISCUSSION

In this study, genetic deletion of *COX-1* led to a decrease in the inflammatory response and neuronal damage in response to $A\beta_{1-42}$, and this effect was associated with alteration of PG production. We show that $A\beta_{1-42}$-induced oxidative damage and degenerating neurons, as well as glial activation, were less severe in *COX-1*$^{-/-}$ mice compared to WT mice. These data suggest that COX-1 facilitates activation of glial cells and supports inflammatory processes and oxidative stress that evolve in neuronal damage, and support previous data from our lab showing that *COX-1*$^{-/-}$ mice have a decreased inflammatory response, oxidative stress and neuronal damage after central injection of LPS [37].

Glial cell activation, in turn, results in enhanced production of a variety of proinflammatory and oxidative mediators, including cytokines, chemokines, and reactive oxygen/nitrogen species [40-42]. Oxidative stress has been recognized to play an important role in

the pathogenesis of AD and linked to the presence of Aβ by the finding of several characteristics, such as enhanced protein, DNA oxidation, and lipid peroxidation in specific regions of the postmortem brain [43-48]. A previous study suggested that oxidative DNA damage reduces the expression of highly vulnerable genes involved in neuronal survival and learning memory, initiating a program of brain aging that starts early in adult life [49]. In addition, lipid peroxidation leads to a reduction in membrane fluidity, alteration of membrane-bounded protein, receptors, and ion channels, and generation of Aβ that induces more oxidative stress and calcium influx that induces glutamate excitotoxicity and cell death [50, 51]. The abundant polyunsaturated lipid content, high oxygen consumption, high metal ion concentration, and low regenerative capacity, as well as relatively low antioxidant levels compared with other tissues make brain highly susceptible to oxidative damage [49, 52]. In addition, oxidative stress differentially affects brain regions, as levels of peroxidizable unsaturated lipids and antioxidant enzymes, and membrane-bound protein differ between brain regions. In this regard, continuous icv infusion of $A\beta_{1-42}$ results in a significant reduction of endogenous antioxidant systems, including Mn-superoxide dismutase (Mn-SOD), glutathione, glutathione peroxidase, and glutathione-S-transferase-π in the hippocampus, cortex, substantia nigra, and thalamus [53]. Importantly, these alterations of each antioxidant enzyme were not uniform, but rather specific in a brain region-dependent manner (e.g. Mn-SOD in CA3), indicating a heterogenous susceptibility to the $A\beta_{1-42}$-induced oxidative stress.

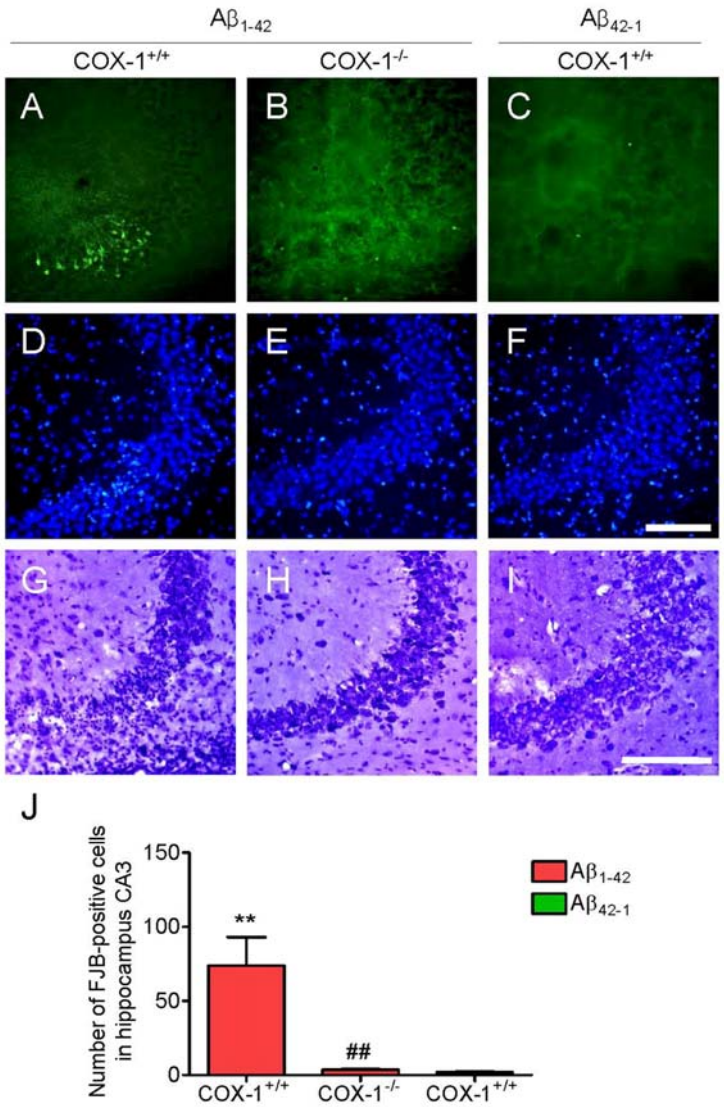

Figure 3. Increased degenerating neurons in the hippocampus 7 d after $A\beta_{1-42}$ administration. (**A-C**) Representative photomicrographs of the CA3 of the hippocampus from WT mice (**A**) injected with $A\beta_{1-42}$ that shows numerous FJB-positive cells compared with $A\beta_{1-42}$–injected *COX-1*$^{-/-}$ mice (**B**). Representative photomicrographs of DAPI (**D-F**) and Nissl staining (**G-I**) in the CA3 of hippocampus from $A\beta_{1-42}$-injected WT (**D, G**) and *COX-1*$^{-/-}$ mice (**E, H**). Scale bar: A-I, 100 μm. (**J**) Comparison of the number of FJB-positive cells from the CA3 area. Mean ± SEM (n = 3-4 per group); $^{**}P < 0.01$ compared with the $A\beta_{42-1}$-injected WT mice; $^{\#\#}P < 0.01$ compared with the $A\beta_{1-42}$-injected WT mice.

Our results show that a single injection of $A\beta_{1-42}$ resulted in a similar spatial distribution of reactive glial cells, nitrotyrosine, and degenerating neurons in the CA3 of hippocampus, suggesting the possibility that glial cell-derived reactive oxygen/nitrogen species may be involved in the impaired neuronal function, which has been described in this model [32, 33, 54, 55]. Indeed, several studies have shown that pretreatment with antioxidants or minocycline, a tetracycline derivative with anti-inflammatory and neuroprotective properties, tend to ameliorate the $A\beta_{1-42}$-induced oxidative damage and behavioral deficits [32,33,56]. Although, variable in terms of the injected Aβ peptide sequences, injection methods, and employed behavioral tests, previous studies have consistently shown the occurrence of behavioral deficits related to memory impairment after intracerebral injection of Aβ peptide [32, 33, 57-59]. Therefore, Aβ injection is a useful *in vivo* model for Aβ toxicity, which is an important component in the progression of AD.

Gene deletion of *COX-1* decreased glial cell activation and attenuated nitrotyrosine induction. The decreased oxidative damage in *COX-1*$^{-/-}$ mice suggests that *COX-1* deletion can reduce the activity of free-radical generating enzymes such as inducible nitric oxide (iNOS), NADPH oxidase, and myeloperoxidase (MPO). These data are consistent with recent observations that genetic deletion of *COX-1* significantly reduces LPS-induced expression of both superoxide (O_2^-) and NO-forming enzymes and thus subsequently attenuates the levels of nitrotyrosine and protein carbonyls, which are considered as biomarkers of oxidative stress [37]. Although, the precise mechanism(s) by which COX-1 regulates free radical-generating enzymes in inflammatory cascade have not been clearly established, it is possible that because of its predominant localization in microglia, COX-1 can modulate the induction of O_2^-, as well as NO, from NADPH oxidase and iNOS, which, in turn, can enhance the production of more potent free radicals such as peroxynitrite ($ONOO^-$). In addition, O_2^- and NO act as potent cell signaling molecules and amplify production of TNF-α and PGE_2 by upregulation of COX-2 [60]. These initial effects combined with the activation of seconddary signaling cascades activate a robust immune response that consequently causes neuronal damage and death.

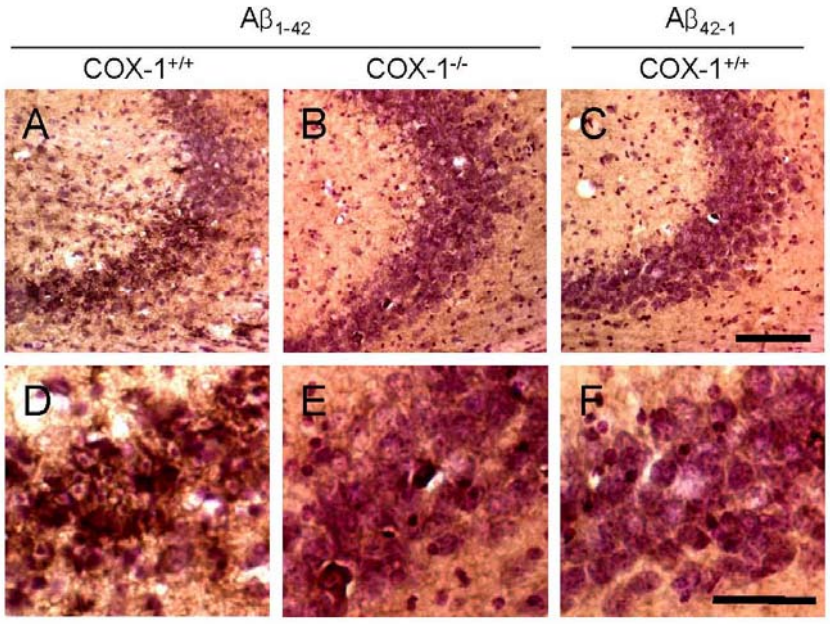

Figure 4. Increased oxidative damage in the hippocampus 7 d after $A\beta_{1-42}$ administration. Representative photomicrographs of the CA1 and CA3 of the hippocampus from WT mice (**A, D**) injected with $A\beta_{1-42}$ that show numerous robustly nitrotyrosine-immunoreactive cells compared with $A\beta_{1-42}$–injected *COX-1*$^{-/-}$ mice (**B, E**). Scale bar: A-C, 100 μm; D-F, 50 μm.

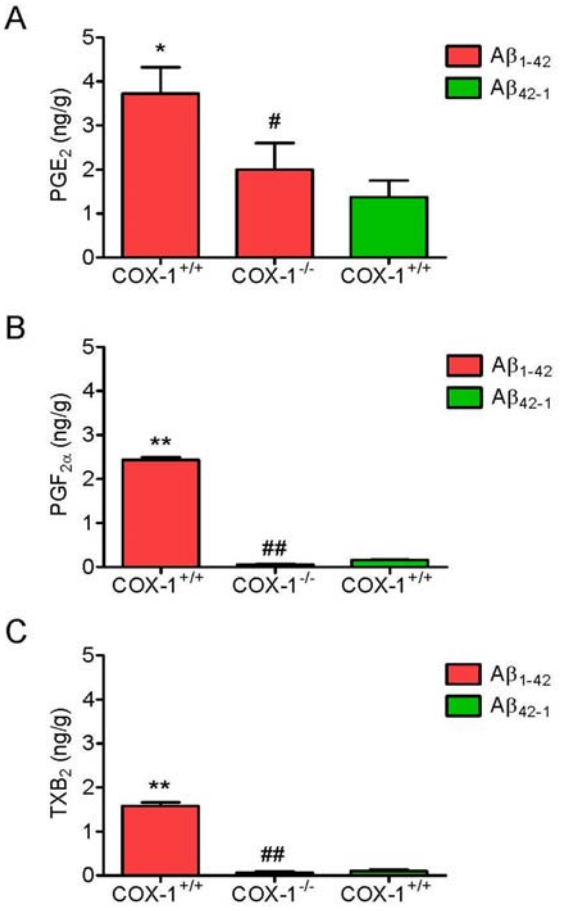

Figure 5. Effects of COX-1 deficiency on PG production 24 h after $A\beta_{1-42}$ administration. $A\beta_{1-42}$–injected WT mice show significantly more PGE_2 (**A**), $PGF_{2\alpha}$ (**B**), and TXB_2 levels (**C**) than *COX-1*$^{-/-}$ mice. Mean ± SEM (n = 3-4 per group); $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the $A\beta_{42-1}$-injected WT mice; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the $A\beta_{1-42}$-injected WT mice.

The results from epidemiological data indicating that NSAIDs are effective in preventing or delaying the onset of AD combined with the failure of COX-2 selective inhibitors in clinical trials in AD patients with moderate to severe AD suggest that either an early treatment is crucial to stop the mechanisms underlying the disease before the onset of the symptoms or that COX-2 selective inhibitors are not effective in delaying the progression of AD. In this regard, an intriguing hypothesis is that the protective effects of NSAIDs may be related to COX-1 rather than COX-2 inhibition. Supporting this hypothesis, COX-1 selective inhibitors (SC-560 and valeryl salicylate), but not COX-2 selective inhibitors (SC-236 and DuP-697), reduce $A\beta_{1-42}$-induced PGs production and neurotoxicity in postmortem human microglia and in murine cortical neurons [61, 62]. Furthermore, a small double blind, placebo-controlled study with indomethacin, a preferential COX-1 inhibitor [63], appeared to protect mild to moderately impaired AD patients from cognitive decline [14]. Interestingly, COX-1 is prominently expressed by microglia in rodent and human brain [2, 4] and appears to be increased in AD brain [2]. Double immunostaining for Aβ and COX-1 indicates clustering of COX-1 positive microglia with classicaland neuritic plaques, although there is no indication that COX-1 is upregulated in activated microglia [64]. However, LPS-induced PGE_2 secretion can be reduced by COX-1 genetic deletion and by COX-1 selective inhibitors [37, 61, 65], suggesting that it is dependent on the constitutive COX-1 activity. In contrast, COX-2 has not been detected in microglia and astrocytes in AD [66]. These combined data suggest that COX-2 may not be the exclusive COX isoform responsible for pathophysiological consequences in neurodegenerative diseases, especially in AD, but that COX-1 also plays a critical role in the process of neuroinflammation and neurodegeneration.

In summary, we show that COX-1 facilitates activation of glial cells and supports inflammatory processes and that genetic deletion of COX-1 significantly attenuates the oxidative stress and neuronal damage in response to $A\beta_{1-42}$. This effect may be due to the predominant localization of COX-1 in microglial cells, where, through its prostaglandin products contributes to the neuroinflammatory cascade of events that ultimately lead to neuronal damage or death. Therefore, COX-1 may represent a viable therapeutic target to treat neuroinflammation and neurodegeneration.

MATERIALS AND METHODS

Animals and stereotaxic $A\beta_{1-42}$ administration. Three-month-old male homozygous *COX-1*$^{-/-}$ and their WT mice (*COX-1*$^{+/+}$) on a C57BL/6-129/Ola genetic background were used [67]. Mice were received at our animal facility at 6 weeks of age from a NIEHS colony maintained by Taconic Farms (Germantown, NY) with heterozygous by heterozygous breedings for greater than 35 generations. In order to prevent the inclusion of strain or genetic background confounders between *COX* null and wild type mice, all of the mice used in this study were progeny derived from heterozygous by heterozygous mating and therefore all contained the same strain and genetic background [67, 68]. The mice were housed at 25°C in our animal facility with a 12 h light/dark cycle with free access to food and water. All animal procedures were approved by the National Institutes of Health (NIH) Animal Care and Use

Committee in accordance with NIH guidelines on the care and use of laboratory animals. $A\beta_{1-42}$ and reverse peptide $A\beta_{42-1}$ (American Peptide, Sunnyvale, CA) were reconstituted in phosphate-buffered saline (pH 7.4) and aggregated by incubation at 37°C for 4 days before use as described previously [69]. $A\beta_{1-42}$ and $A\beta_{42-1}$ (400 pmol per mouse) were administered intracerebroventricularly (i.c.v) into the lateral ventricle using a 10 μl syringe with a fine needle (World Precision Instruments, Sarasota, FL) and a syringe pump (Stoelting, Wood Dale, IL) at a rate of 1 μl/min. The dose of $A\beta_{1-42}$ and $A\beta_{42-1}$ was selected based on previous studies [32-35]. The coordinates for the stereotaxic injections were –2.3 mm dorsal/ventral, –1.0 mm lateral, and –0.5 mm anterior/posterior from the bregma [70].

Tissue preparation and histology. Mice were transcardially perfused with saline followed by 4% paraformaldehyde. Brains were postfixed overnight in the same medium and placed in 30% sucrose, before sectioning (30 μm). Immunohistochemistry and double immunofluorescence were performed as described previously [71]. Rabbit anti-IBA-1 (1:500; Wako), mouse anti-GFAP (1:200; Sigma-Aldrich), and mouse anti-nitrotyrosine (1:100; Chemicon, Temecula, CA) were used as primary antibodies. The slides were visualized by brightfield microscopy (Olympus) and digitally photographed. FJB, a fluorochrome for the sensitive histochemical localization of neuronal degeneration, was used to identify degenerating neurons [72]. Brain sections were mounted on gelatin-coated slides and completely dried. Then sections were rehydrated through graded concentrations of alcohol (100, 70, and 50%; 1 min each), and rinsed for 1 min in distillated water. The slides were incubated in a solution of 0.06% potassium permanganate for 20 min, rinsed in distilled water for 1 min, and transferred to FJB (Histochem, Jefferson, AR) staining solution (0.001% FJB/0.1% acetic acid) for 20 min. The slides were thereafter rinsed three times in distilled water and air dried then immersed in xylene and coverslipped with mounting media. The slides were visualized by fluorescent microscopy (Olympus) and digitally photographed. Because the FJB staining was obvious on digital imaging, the number of FJB-positive cells per section was quantified as described previously [73]. The number of microglia per section was quantified by counting the number of IBA-1-stained cell bodies within 0.3 mm^2 area of the CA3. For each measurement, two blinded independent investigators counted 3-4 brains per group, 3 sections per brain.

Measurement of prostanoids. Prostanoids were purified from the lipid extract as previously described [74] and levels were determined using specific enzyme immunoassay (EIA) kits, PGE_2, $PGF_{2\alpha}$, and TXB_2, (Oxford Biomedical, Oxford, MI).

Statistics. All data are expressed as mean ± SEM. Statistical significance was assessed with one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test using GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA). Significance was taken at $P < 0.05$.

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CONFLICT OF INTERESTS STATEMENT

The authors have no conflict of interest to declare.

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