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Neuronal overexpression of cyclooxygenase-2 does not alter the neuroinflammatory response during brain innate immune activation

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Abstract

Neuroinflammation is a critical component in the progression of several neurological and neurodegenerative diseases and cyclooxygenases (COX) -1 and -2 are key regulators of innate immune responses. We recently demonstrated that COX-1 deletion attenuates, whereas COX-2 deletion enhances, the neuroinflammatory response, blood-brain barrier permeability and leukocyte recruitment during lipopolysaccharide (LPS)-induced innate immune activation. Here, we used transgenic mice, which overexpressed human COX-2 via neuron-specific Thy-1 promoter (TgCOX-2), causing elevated prostaglandins (PGs) levels. We tested whether neuronal COX-2 overexpression affects the glial response to a single intracerebroventricular injection of LPS, which produces a robust neuroinflammatory reaction.

Relative to non transgenic controls (NTg), 7 month-old TgCOX2 did not show any basal neuroinflammation, as assessed by gene expression of markers of inflammation and oxidative stress, neuronal damage, as assessed by Fluoro-JadeB staining, or systemic inflammation, as assessed by plasma levels of IL-1 β and corticosterone. Twenty-four hours after LPS injection, all mice showed increased 1) microglial activation, as indicated by Iba1 immunostaining, 2) neuronal damage, 3) mRNA expression of cytokines (TNF- α , IL-6), reactive oxygen expressing enzymes (iNOS and NADPH oxidase subunits), endogenous COX-2, cPLA₂ and mPGES-1, 4) hippocampal and cortical IL-1 β levels. However, the increases were similar in TgCOX-2 and NTg. In NTg, LPS increased brain PGE₂ to the levels observed in TgCOX2. These results suggest that PGs derived from neuronal COX-2 do not play a role in the neuroinflammatory response to acute activation of brain innate immunity. This is likely due to the direct effect of LPS on glial rather than neuronal cells.

Keywords

Cyclooxygenase-2; microglia; neuron; neuroinflammation; cytokines; lipopolysaccharide

Cyclooxygenases (COX)-1 and -2 catalyze the first committed reaction in the metabolism of arachidonic acid (AA) to bioactive prostaglandins (PGs) [27]. Both COX-1 and COX-2 are constitutively expressed in the brain, where COX-1 is mainly expressed in microglia [15], whereas COX-2 is localized in postsynaptic dendrites and excitatory terminals with both

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neuronal and vascular associations [38]. In the central nervous system (CNS), COX-2 has been implicated in important physiological functions such as synaptic transmission, neurotransmitter release, blood flow regulation, and sleep/wake cycle [13,17,24,28,34].

COX-2 expression is upregulated in a variety of neuropathological diseases with a marked inflammatory component such as multiple sclerosis, Parkinson's disease, and in the early stages of Alzheimer's disease (AD), [14,15,21]. Furthermore, epidemiological studies showed that prolonged use of non-steroidal anti-inflammatory drugs (NSAIDs) delays the onset and reduces the risk of AD [5,22,30]. However, clinical studies using selective COX-2 inhibitors have been unsuccessful in delaying AD progression, except for a small trial using indomethacin, a COX-1 preferential inhibitor [31]. Some authors concluded that COX-2 may not be the molecular target of the efficacy of NSAIDs [21].

COX-1 and COX-2 isoforms play distinct roles during neuroinflammation [7]. We previously demonstrated that genetic deletion of COX-1 attenuates, whereas genetic deletion of COX-2 enhances, the neuroinflammatory response, blood-brain barrier permeability and leukocyte recruitment during (LPS)-induced innate immune activation [2,3,8,9]. We suggested that, because of its predominant localization in microglia, COX-1 may be the major player during endotoxin-induced neuroinflammation, and COX-2-derived products may be critical for timely resolution and termination of inflammation [12,19]. Previous reports indicate that transgenic mice overexpressing neuronal COX-2 have altered fever response [37] and upregulated mRNA expression of complement component C1qB in the dentate gyrus, CA3 and CA2 areas [33], suggesting those mice may respond differently to inflammation. COX-2 overexpressing mice also had a significant increase in infarct volume after middle cerebral artery occlusion as compared with nontransgenic littermates [11]. In this study we investigated whether a constitutive overexpression of neuronal COX-2 would alter the glial response to LPS.

Animals

Pathogen free 5–7 month-old male C57B6/J/transgenic COX-2 mice of 300 line (TgCOX2) and non-transgenic littermate (NTg) were used. To generate the transgenic mice, the human COX-2 ORF was subcloned into the second exon of the Thy-1 promoter as described previously [4]. As a result, this transgenic line can exhibit up to 10-fold increase in brain PGE₂ levels [37]. Mice were housed at 25 °C in a NIH animal facility with a 12 h light/dark cycle with free access to food and water. All procedures were performed under a NIH approved animal protocol in accordance with the NIH guidelines on the care and use of laboratory animals.

LPS administration

Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg, i.p.) and positioned in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA). Vehicle (sterile phosphate buffered saline, 5 µl) or LPS (*E. coli* serotype 055:B5 (Sigma); 5 µg in 5 µl of sterile PBS) was administered into the cerebral lateral ventricle (stereotaxic coordinates: –2.3 mm dorsal/ventral, –1.0 mm lateral, and –0.5 mm anterior/posterior from the bregma [29]) as previously described. This dose of LPS and this time point (24 h) have been shown by us and by other groups to produce a robust neuroinflammatory response [2,3,9,10,23]. Mice were sacrificed 24 h after LPS injection, the brain was removed and the hippocampus and cerebral cortex were dissected out on ice, then snap frozen in methyl-butane (–60 °C) and stored at –80 °C for further experiments. After decapitation, trunk blood was collected into lithium-heparin coated tubes (BD, NJ, USA), centrifuged (1000 rpm for 10 min) and the plasma collected and stored at –80°C.

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 μ m). Degenerating neurons were identified using Fluoro-Jade B (FJB), a fluorochrome for the sensitive histochemical localization of neuronal degeneration [32] as previously described [2]. FJB staining in the hippocampal area was quantified using a pathology index (1=mild pathology, 2=moderate pathology, 3=severe pathology) [36]. Immunohistochemistry was performed as previously described [2]. Rabbit anti-iba-1 (1:500; Wako) primary antibody was used as a microglial marker.

Western blotting

The cerebral cortex was homogenized in 10 mM HEPES, pH 7.9, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 10 mM KCl, buffer with a protease inhibitor cocktail (Roche) using a Teflon-glass homogenizer. After adding 0.5% igeal CA630 (Sigma), five additional strokes of homogenization were performed. After centrifugation at $13,000 \times g$ (4 °C), the supernatant was collected and assayed for Western blot analyses as previously described [2] using the following primary antibodies: p67^{phox} (1:500; BD Biosciences), iNOS (1:250; Upstate, USA), COX-1 (1:500; Cayman Chemicals), and glyceraldehyde dehydrogenase (Gapdh, 1: 2000; Santa Cruz, CA) to control for protein loading. Blotted proteins were detected and quantified using an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NB).

IL-1 β and corticosterone measurements

For IL-1 β measurement, an aliquot of the crude homogenate for both hippocampus and cerebral cortex was centrifuged at $10,000 \times g$ for 20 min at 4 °C. Brain (pg/mg protein) and plasma IL-1 β (pg/ml; R&D Biosystems, Minneapolis, MN) and corticosterone (pg/ml; Cayman chemicals, Ann Arbor, MI) were assayed using ELISA-based kits.

Brain PGE₂ levels

the whole brain was extracted using hexane: 2-propanol (3:2, v/v) as previously described [1] and then assayed using ELISA-based kit (Cayman chemicals, Ann Arbor, MI).

Quantitative real-time PCR

Brain total RNA was extracted and reverse transcribed as previously described [1]. Quantitative real-time PCR for glial fibrillary acidic protein (GFAP), scavenger receptor A (SRA), cluster of differentiation 11b and 45 (CD11b, CD45), tumor necrosis factor alpha (TNF- α), interleukin 1 β and 6 (IL-1 β , IL6), inducible and endothelial nitric oxide synthases (iNOS, eNOS), the NADPH oxidase subunits gp91^{phox} and p67^{phox}, microsomal prostaglandin synthase1 (mPGES-1), cytosolic phospholipase 2 (cPLA₂), mouse COX-1, mouse and human COX-2 was performed using the Assay-On-Demand Gene Expression protocol as previously described [1]. Data were analyzed using the comparative threshold cycle (Ct) method [20], and results were normalized with phosphoglyceratekinase 1 (Pgk1) as an endogenous control and expressed as fold difference from vehicle-injected NTg.

Statistics

Data were expressed as mean $\pm$ SEM and analyzed with a two-way ANOVA (genotype and treatment) or a student t-test (2 groups) using SigmaPlot for Windows, version 11.0 (Systat software, Germany). To normalize values, data were log-transformed before analysis. Chi-

Square test was used for statistical analysis of pathology index. P values < 0.05 were considered statistically significant.

To test whether mice overexpressing COX-2 had increased basal inflammation, we measured brain mRNA expression of several markers of inflammation and oxidative stress, as well as plasma levels of IL-1 β and corticosterone in unchallenged NTg and TgCOX-2 mice. Plasma levels of IL-1 β and corticosterone were similar in naïve unchallenged NTg and TgCOX2 mice (IL-1 β : 7.2 $\pm$ 5.4 for NTg (n=4) and 4.0 $\pm$ 1.5 pg/ml for TgCOX2 (n=4); corticosterone: 11.7 $\pm$ 4.8 for NTg (n=6) and 11.5 $\pm$ 3.9 pg/ml for TgCOX2 (n=6)). Gene expression of TNF- α , IL-1 β , CD45, CD11b, GFAP, cPLA2, COX-1, COX-2, iNOS, eNOS, gp91^{phox}, and p67^{phox} were similar between TgCOX2 and NTg mice (Fig. 1A). These data suggest that constitutive overexpression of neuronal COX-2 does not affect basal brain or systemic inflammation.

We assessed neuronal damage in the brain at 24 h after LPS injection using FJB, which selectively labels injured neurons [32]. The occurrence of FJB-positive cells in the hippocampal formation was similar in NTg and TgCOX2 (Fig. 1B–D). To determine whether TgCOX-2 mice had an altered glial response, we assessed microglial activation in the brain using Iba-1 as a microglial marker. Intense Iba1-positive microglia with enhanced staining intensity, enlarged cell bodies, and ramified processes were observed 24 h after LPS injection in the cortical/caudate putamen and hippocampal area of all mice. However, no significant difference was observed between TgCOX2 and NTg (Fig. 1E–H). Vehicle injected TgCOX2 mice have 6.5 fold more brain PGE₂ compared to NTg (Fig. 1I). This is in agreement with the original report by [4,37] which reported up to 10 fold increase in brain PGE₂ levels in TgCOX2 mice compared to NTg mice. In NTg mice, LPS increased brain PGE₂ levels 7 fold compared to vehicle, whereas LPS does not significantly increase brain PGE₂ levels in the TgCOX2 mice (Fig. 1I). These data suggest that the constitutive presence of PGE₂ and other COX-2 products prevent the further upregulation of brain PGE₂ levels by LPS in the TgCOX2.

Brain protein levels of the pro-inflammatory cytokine IL-1 β were similarly increased after LPS in TgCOX2 and NTg mice (hippocampal IL-1 β : 105.6 $\pm$ 28.9 pg/mg protein for NTg (n=4) and 138.1 $\pm$ 46.1 pg/mg protein for TgCOX2 (n=5); cortical IL-1 β : 13.4 $\pm$ 1.8 pg/mg protein for NTg (n=4) and 13.4 $\pm$ 4.8 pg/mg protein for TgCOX2 (n=5)).

To further assess the inflammatory response in TgCOX-2 and NTg mice, we measured brain mRNA expression of cytokine and glial markers, 24 h after LPS injection. Brain mRNA expression of the cytokines IL-6 and TNF- α , and of microglial (SRA) and astrocytic (GFAP) markers were increased similarly after LPS in TgCOX2 and NTg mice (Fig. 2A; p > 0.05). iNOS, eNOS, and NADPH oxidase, major sources of reactive oxygen species during inflammation, are expressed in glial cells and may be involved in the neuroinflammatory injury. After LPS injection, mRNA of iNOS, eNOS and NADPH oxidase subunits (gp91^{phox} and p67^{phox}) as well as protein expression of iNOS and p67^{phox} were increased similarly in the brain of TgCOX2 and NTg mice (Fig. 2B–C). Brain transcripts of cPLA₂, COX-2 and mPGES-1 (Fig. 2D) were similarly upregulated after LPS in NTg and in TgCOX2 mice (p > 0.05). COX-1 mRNA and protein expression remained unchanged in all groups (Fig. 2D–E). The expression of the human COX-2 transgene was not affected by LPS treatment (**data not shown**). We also assessed the gene expression of several markers of inflammation and oxidative stress at an earlier time point (6h) (Fig. 2F). The gene expression of TNF- α , IL-1 β , CD45, CD11b, GFAP, cPLA₂, COX-1, COX-2, iNOS, eNOS and gp91^{phox} was not affected by LPS at 6 h, except for p67^{phox} mRNA that was significantly decreased in the TgCOX2 brain compared to NTg brain after LPS (Fig. 2F; 28%, p = 0.01, t-test).

Overall, our data show that neuronal overexpression of COX-2 did not modify the neuroinflammatory response to LPS and caused similar neuronal damage and glial activation,

brain level of IL-1 β , and gene expression of several inflammatory markers compared to non transgenic mice. We previously demonstrated that genetic deletion of COX-2 exacerbates the inflammatory response and neuronal damage to centrally injected LPS. In particular, we showed that after LPS brain IL-1 β and NADPH oxidase subunit p67^{phox} were upregulated in COX-2 deficient mice and mice treated chronically with celecoxib, a selective COX-2 inhibitor [2].

In this study, human COX-2 was specifically overexpressed in neurons, whereas in our previous study, COX-2 gene was deficient in all cell types in the knockout mice. Previous work showed that the expression of transgenic COX-2 has a global pan-neuronal pattern, with the exception of the dentate and the striatum areas [37]. Moreover, TgCOX2 mice demonstrated increased level of PGE₂, PGD₂, PGI₂, PGF_{2 α} , and TBX₂, with a dominant production of PGE₂, followed by PGI₂, over the other products [37]. It has been previously shown that, compared to NTg, TgCOX2 mice exhibited cognitive deficits at 12 months of age, and increased neuronal apoptosis and astrocytic activation at 20 months [4]. However, at 7 months, approximately the same age as the mice used in our study, transgenic mice exhibited intact learning ability and memory retention. Under basal conditions, we showed here that TgCOX2 mice did not exhibit any neuronal damage as assessed by FJB staining (data not shown), and no obvious inflammatory pattern as assessed by levels of plasma IL-1 β and corticosterone and brain mRNA expression of several markers of inflammation and oxidative stress. After LPS challenge, brain PGE₂ in NTg mice was increased to the levels of TgCOX-2 mice, for which brain PGE₂ were not changed after LPS. This suggests that the pre-existing overproduction of PGE₂, and possibly others COX-2 products, by neurons does not alter the inflammatory response nor increase the susceptibility to LPS, whereas reduced levels of COX-2-derived products in the COX-2 deficient mice caused an increased inflammatory response to LPS, probably through glial activation. Indeed, LPS, a cell wall component of Gram-negative bacteria, binds to CD14 protein and potently activates the toll-like receptor 4, which is expressed *in vivo* by microglia but not neurons [18].

A conflicting view exists on the role of COX-2 in cell survival and responses in the brain depending on the type of insult, the time of insult and the tissue examined [24]. Several studies demonstrated an important role of COX-2 in ischemic neuronal death. Indeed, pharmacological inhibition or genetic deletion of COX-2 alleviates neuronal damage after ischemia [6,16,25, 26], whereas neuronal overexpression of hCOX-2 in transgenic mouse model potentiates neuronal injury after global ischemic insult [40]. However, using COX-2 deficient mice or selective COX-2 inhibitors, our and other groups demonstrated that COX-2 has a neuroprotective role during acute neuroinflammation [7]. In the normal brain, COX-2 is constitutively expressed by excitatory neurons at post-synaptic sites in brain and regulated by synaptic activity [17,41]. Although, LPS induced a clear inflammatory response, as indicated by the increased mRNA expression of TNF- α , IL-6, terminal prostaglandin synthase mPGES-1, and the ROS-producing enzymes (iNOS, eNOS, NADPH oxidase), an increased expression of neuronal COX-2 did not change this response. Therefore, these results suggest that PGs or other neuronal COX-2-derived bioactive products are not major players during endotoxin activation of glial innate immunity. In agreement with this hypothesis, models where the toxins directly damage neurons, such as MPTP (1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine) induced damage, the neuroprotective effect of COX-2 inhibition does not appear to be linked to the inflammatory response [35].

In conclusion, eicosanoids derived from neuronal COX-2 do not contribute to the inflammatory response during direct activation of glial innate immunity, whereas they can potentiate damage in injury models that directly challenge neurons. These data support the hypothesis that toxic or protective effects of COX-2 derived prostanoid signaling on neuronal viability depends on

whether the primary stimulus is inflammatory or excitotoxic and on the cell type targeted (neuron or glia) [7,39].

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Figure 1.

Effects of overexpression of human COX-2 in neuron on basal expression of inflammatory and oxidative stress markers, LPS-induced neuronal degeneration and microglial activation and PGE $_2$ production. **A)** Quantitative real time-PCR analysis of *cPLA $_2$* , *COX-1*, *COX-2*, the cytokines *IL-1 β* and *TNF- α* , microglia/macrophage markers *CD11b* and *CD45*, astrocyte marker *GFAP*, the oxidative stress markers *iNOS*, *eNOS*, *gp91^{phox}* and *p67^{phox}* in the brain of naïve unchallenged TgCOX2 (n=9) and NTg mice (n=11). **B–C)** Representative photomicrographs of LPS-induced neuronal degeneration using FJB staining in the hippocampus of TgCOX2 and NTg mice 24 h after icv LPS. Bars represent 100 μ m. **D)** Quantification of FJB staining in the hippocampus of TgCOX2 and NTg mice 24 h after LPS,

using an ordinal pathology index: 1=mild pathology; 2=moderate pathology; 3=severe pathology. Data are expressed as frequency of FJB positive cells in function of pathology index. **E–H)** Representative photomicrographs of LPS-induced microglial activation using Iba1 immunohistochemistry in the hippocampal area 24 h after icv LPS. Photomicrographs **F** and **H** focused on the CA1 hippocampal fields with 60x magnification. Bars represent 100 μ m. **I)** Brain PGE₂ levels in the TgCOX2 and NTg mice 24 h after icv LPS or vehicle. Data were analyzed with two-way ANOVA (NTg vehicle=3; NTg LPS=5, TgCOX2 vehicle=4; TgCOX2 LPS = 5). ***p<0.01 vs. vehicle-injected mice (LPS effect); ###p<0.01 vs. vehicle-injected NTg mice (genotype effect). Data are presented as mean $\pm$ SEM.

Figure 2.

Effects of overexpression of human COX-2 in neuron on LPS-induced expression of brain inflammatory and oxidative stress markers. **A**) Quantitative real time-PCR analysis of cytokines (*IL-6*, *TNF- α*) microglia marker *SRA1* and astrocyte marker *GFAP* in TgCOX2 and NTg mice ($n = 5-7$). 24 h after icv LPS or vehicle. *** $P < 0.001$ compared to the corresponding vehicle-injected mice. **B**) Quantitative real time-PCR analysis of ROS-expressing enzymes (*iNOS*, *eNOS*, *gp91^{phox}* and *p67^{phox}*) in NTg mice and TgCOX2 mice 24 h after icv LPS or vehicle. **C**) Representative immunoblots of *iNOS* and *p67^{phox}* expression in the cerebral cortex of TgCOX2 and NTg mice 24 h after LPS or vehicle. **D**) Quantitative real time-PCR analysis of enzymes involved in the arachidonic acid cascade (*cPLA₂*, *COX-2*, *COX-1*, *mPGES-1*) in

TgCOX2 and NTg mice ($n = 5-7$) 24 h after icv LPS or vehicle. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared to the corresponding vehicle-injected mice. **E)** Representative immunoblot of COX-1 expression in the cerebral cortex of TgCOX2 and NTg mice 24 h after LPS or vehicle. **F)** Quantitative real time-PCR analysis of *TNF- α* , *IL-1 β* , *CD11b*, *CD45*, *GFAP*, *cPLA2*, *COX-1*, *COX-2*, *iNOS*, *eNOS*, *gp91^{phox}* and *p67^{phox}* in TgCOX2 ($n=6$) and NTg ($n=7$) mice 6 h after icv LPS or vehicle. $**P=0.01$ (T' Test). Data are presented as mean $\pm$ SEM.