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Interleukin-13 Enhances Cyclooxygenase-2 Expression in Activated Rat Brain Microglia: Implications for Death of Activated Microglia

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Brain inflammation has recently attracted widespread interest because it is a risk factor for the onset and progression of brain diseases. In this study, we report that cyclooxygenase-2 (COX-2) plays a key role in the resolution of brain inflammation by inducing the death of microglia. We previously reported that IL-13, an anti-inflammatory cytokine, induced the death of activated microglia. These results revealed that IL-13 significantly enhanced COX-2 expression and production of PGE2 and 15-deoxy-

A

Although a major role of inflammation is host defense against bacterial and viral infection of injured tissue, inflammatory mediators can also damage the surrounding tissue and aggravate injury. In fact, brain inflammation aggravates ischemic brain damage (1) and has been considered a risk factor for Alzheimer’s and Parkinson’s diseases (2, 3). Thus, endogenous mechanisms should exist for controlling the duration and extent of inflammation.

In injured brain, microglia, which are the resident brain macrophages, can be activated by factors released from damaged cells or by infiltrating blood components following damage to the blood-brain barrier (4–6). However, microglia are not observed at injured sites several days after injury (7). Apoptosis appears to be involved in clearing activated microglia (8, 9). Therefore, death of activated microglia could act as an endogenous mechanism for the resolution of brain inflammation.

There is evidence that an anti-inflammatory cytokine, IL-13, may be involved in the resolution of brain inflammation. It is generally accepted that anti-inflammatory cytokines down-regulate monocyte/macrophage production of proinflammatory mediators, including reactive oxygen species, nitrogen intermediates, IL-1β, IL-6, IL-8, and TNF-α (10, 11). In previous studies, we found that IL-13 induced the death of activated microglia in vitro and in vivo (8, 9). Furthermore, activation and survival of rat brain microglia are prolonged by coinjection of IL-13-blocking Abs with LPS compared with LPS alone (9). However, the question of how IL-13 induces the death of activated microglia has not been resolved.

In the present study, we show that IL-13 enhances cyclooxygenase-2 (COX-2) expression and the production of PGE2 and 15d-PGJ2 in LPS-treated microglia. This function of IL-13 is not mimicked by other anti-inflammatory cytokines such as IL-10 or TGF-β. More importantly, the COX-2 products, PGE2 and 15d-PGJ2, appear to be involved in IL-13-induced death of activated microglia.

Materials and Methods

Reagents

LPS and thrombin were purchased from Sigma-Aldrich. IL-13, IL-4, IL-10, and TGF-β were from PeproTech. PGE2 was from Sigma-Aldrich.

‡Abbreviations used in this paper: COX-2, cyclooxygenase-2; 15d-PGJ2, 15-deoxy-

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Sulprostone, butaprost, AH6809, and 11-deoxy PGE₂ were from Cayman Chemical. Ciglitazone, 15d-PGJ₂, and SP600125 were from BIOMOL. GW9662 was from Tocris Cookson. NS398 was from Calbiochem. Abs against COX-1, COX-2, and JNK were from Santa Cruz Biotechnology, and Abs against phospho-JNK, phospho-ERK, and phospho-p38 were from Cell Signaling Technology. Reverse transcriptase was from Promega. Primers for PCR were from Bioneer. Calcein-acetoxyethyl ester (AM) and ethidium homodimer-1 (Etd-1) for measurement of cell death were from Molecular Probes. Enzyme immunoassay kits for PGE₂ and 15d-PGJ₂ were from Amersham Biosciences and Assay Designs, respectively.

**Cell culture**

Microglia were prepared from the cerebral cortices of 1- to 3-day-old Sprague-Dawley rats, as previously described (4, 12). Briefly, cortices were triturated into a single cell suspension in MEM containing 10% FBS and incubated for 2 wk. Microglia were then detached from flasks by mild shaking and filtered through a nylon mesh to remove astrocytes and clumped cells. Cells were seeded in plates or dishes and washed 1 h later to remove unattached cells. Primary astrocytes remaining in the flask were harvested with 0.1% trypsin. Astrocytes were plated in plates and cultured in MEM supplemented with 5% FBS.

**RT-PCR**

Total RNA was extracted using RNazol, and cDNA was prepared using reverse transcriptase, according to the manufacturer’s instructions. The sequences of PCR primers were as follows: forward, 5′-ACACTCTATGTTTGAGACCTTCAACACCCC-3′ and reverse, 5′-GAAGGGGACACCCCTTCATCAT-3′ for COX-2; forward, 5′-GATGCCAACGCTGTCGAACAAA-3′ and reverse, 5′-CCCTTCTCCAGCTGGGAGAC-3′ for TNF-α; and forward, 5′-CATGTGGAGACTTCAACCACCCC-3′ and reverse, 5′-GCGATCTCTCTGCCGAAATCCTAG-3′ for actin. PCR products were separated by electrophoresis in 1.5% agarose gels and detected under UV light.

**Western blot analysis**

Cells were lysed in 50 mM Tris-HCl (pH 7.4) containing 1% Nonidet P-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM Na₂VO₃, 1 mM NaF, and protease inhibitors. The proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and visualized using primary Abs, followed by peroxidase-conjugated secondary Abs and ECL system.

**PG assay**

PGE₂ (or 15d-PGJ₂) in culture medium was assayed using an enzyme immunoassay kit following the manufacturer’s protocol. The assay is based on the competition between peroxidase (or alkaline phosphate)-conjugated tracer PGE₂ (or 15d-PGJ₂) and PGE₂ (or 15d-PGJ₂) in the medium for a limited number of PGE₂ (or 15d-PGJ₂)-specific Abs. The amount of remaining tracer PGE₂ (or 15d-PGJ₂) was determined by addition of substrates for peroxidase (or alkaline phosphatase). ODs were determined at 405 (or 450) nm.

**Measurement of cell death**

Viability of microglia was assessed by double labeling of cells with 2 μM calcein-AM and 4 μM Etd-1. Calcein-positive live cells and ethidium-positive dead cells were counted in eight fields from two different wells for each group using a fluorescence microscope (Nikon Diaphot 300).

**Statistical analysis**

Data were analyzed by t test or one-way ANOVA, followed by post hoc comparisons (Student-Newman-Keuls) using the Statistical Package for Social Sciences 8.0 (SPSS).

**Results**

**IL-13 enhances COX-2 expression in microglia treated with LPS**

Although IL-13 is a well-known anti-inflammatory cytokine, the effect of IL-13 on brain inflammation is relatively unknown. Therefore, we examined the effect of IL-13 on the expression of TNF-α and COX-2 in LPS-treated microglia by RT-PCR because TNF-α and COX-2 are important inflammatory mediators. Upon LPS treatment, TNF-α and COX-2 mRNA expression increased within 3–6 h, and their expression was sustained for up to 3 days (data not shown). When cells were treated with LPS in the presence of IL-13, the LPS-induced expression of TNF-α mRNA was reduced (Fig. 1A). Unexpectedly, however, IL-13 significantly enhanced LPS-induced expression of COX-2 mRNA (Fig. 1A). Consistent with these findings, Western blot analysis showed enhanced COX-2 protein expression in cells cotreated with LPS and IL-13 at 24 h and a further enhancement at 72 h, whereas IL-13 alone had no effect on the levels of COX-2 (Fig. 1B). As expected, the expression of COX-1, a constitutively expressed isoform of COX, was not changed by LPS or IL-13 treatment (Fig. 1C). We also found that IL-13 enhanced COX-2 expression in cells treated with thrombin, another activator of microglia (Fig. 1D). Collectively, these results indicate that IL-13 enhances COX-2 expression in activated microglia irrespective of the kind of stimulator.

**IL-4, but neither IL-10 nor TGF-β, enhances COX-2 expression in microglia treated with LPS**

Next, we investigated whether other anti-inflammatory cytokines affected COX-2 expression in a manner similar to IL-13. Like IL-13, we found that IL-10 and TGF-β reduced LPS-stimulated expression of TNF-α mRNA, but neither affected the LPS-induced expression of COX-2 mRNA (Fig. 2A). Similar results were observed at the protein level in that both IL-10 and TGF-β barely enhanced COX-2 expression (Fig. 2B). However, IL-4, which shares the IL-13R, mimicked the ability of IL-13 to enhance LPS-stimulated expression of COX-2 protein (Fig. 2C). We further examined the PG levels in the absence or presence of IL-13, IL-10, or TGF-β. Although both PGE₂ and 15d-PGJ₂ are produced by the enzymatic action of COX-2, they are considered to be pro- and anti-inflammatory PGs, respectively (13–15). Therefore, we measured the amount of both PGE₂ and 15d-PGJ₂. Cells were incubated with 10 ng/ml LPS in the presence or absence of IL-13, IL-10, or TGF-β for 3 days. LPS alone resulted in the production of 0.56 ± 0.04 nM PGE₂ and 2.62 ± 0.44 nM 15d-PGJ₂. Addition of IL-13 along with the LPS enhanced the PGE₂ levels by ~28-fold (16.39 ± 2.53 nM) and 15d-PGJ₂ by ~7-fold (17.92 ± 3.23 nM) compared with LPS alone (Fig. 2, D and E). We also examined the effect of IL-10 and TGF-β on LPS-stimulated PGE₂ and 15d-PGJ₂ production. Neither IL-10 nor TGF-β significantly changed PGE₂ (0.72 ± 0.26 nM and 1.30 ± 0.55 nM, respectively) or 15d-PGJ₂ production (1.55 ± 0.06 nM and 1.43 ± 0.24 nM, respectively) (Fig. 2, D and E). The amount of PGE₂ and 15d-PGJ₂ from cells untreated or treated with each cytokine alone produced...
<0.11 nM and 0.83 nM, respectively. Taken together, these results suggest that IL-13 enhancement of LPS-stimulated COX-2 expression and PGE2 and 15d-PGJ2 production is not a general function of anti-inflammatory cytokines.

**IL-13-enhanced COX-2 expression mediates microglial death**

Previously, we reported that IL-13 and IL-4, but not TGF-β, induced the death of activated microglia (8, 9). We also found that IL-10 had no effect on microglial death (data not shown). These findings together with the present results indicate that IL-13 and IL-4 enhance COX-2 expression and induce the death of activated microglia, whereas IL-10 and TGF-β have little effect on either COX-2 expression or microglial death. Therefore, we examined the possible association between COX-2 expression and the death of activated microglia. For this, we tested the effect of COX inhibitors on IL-13-induced death of activated microglia using calcein-AM and Etd-1. Fluorescence micrographs were obtained using a Nikon Diaphot 300 (A). The number of Etd-1-positive dead cells was counted, as in B. Values in B and C are the means ± SEM of eight replicates. The data are representative of more than three independent experiments.

**EP2 and peroxisome proliferator-activated receptor (PPAR) γ mediate IL-13-induced death of activated microglia**

Given the finding that COX-2 inhibitors reduced IL-13-induced microglial death, we suspected that the COX-2 products, PGE2 and 15d-PGJ2, may mediate microglial death. We found that the addition of PGE2 (0.01–1 µM) or 15d-PGJ2 (0.01–5 µM) caused dose-dependent death of microglia within 3 days (Fig. 4A). In addition, the effect of PGE2 and 15d-PGJ2 on cell death was additive when cells were treated with combination of these two PGs (Fig. 4A). We also tested the effect of PGE2 and 15d-PGJ2 on astrocytes, and we found that neither induced death in astrocytes at the concentrations that were cytotoxic to microglia (Fig. 4B). These results suggest IL-13-enhanced COX-2 expression resulted in increased PGE2 and 15d-PGJ2 production and that these PGs selectively promote the death of microglia, but not astrocytes.

Next, we examined the receptor subtypes involved in PGE2- and 15d-PGJ2-induced microglial death. Microglia have been shown to express all four PGE receptors (EP1, EP2, EP3, and EP4) as well as the 15d-PGJ2 receptor, PPARγ (13, 16–19). Microglia were treated with 11-deoxy PGE1 (EP2 and EP4 agonist; 0.001–10 µM), butaprost (EP2-selective agonist; 0.001–10 µM), sulprostone (EP1 and EP3 agonist; 0.001–10 µM), or ciglitazone (PPARγ agonist; 0.01–10 µM), and cell death was assayed after 3 days.

![FIGURE 2](image-url)

**FIGURE 2.** IL-4, but not IL-10 or TGF-β, mimics the effect of IL-13 on COX-2 expression and PGE2/15d-PGJ2 production. Microglia were co-treated with 10 ng/ml LPS and IL-10 (20 ng/ml), IL-13 (20 ng/ml), TGF-β (10 ng/ml), or IL-4 (20 ng/ml), as indicated. Expression of TNF-α and COX-2 mRNA was detected by RT-PCR at 72 h (A), and COX-2 expression was detected by Western blot at 72 h (B) or at the indicated times (C). Actin levels were measured as an internal control in RT-PCR (A), and JNK expression was measured as a loading control in Western blots (B and C). The amount of PGE2 (D) or 15d-PGJ2 (E) was measured by enzyme immunoassay at 72 h. Values are means ± SEM of three (PGE2) or seven (PGJ2) samples. *, p < 0.05, compared with untreated control. #, p < 0.01, compared with LPS.

![FIGURE 3](image-url)

**FIGURE 3.** COX-2 mediates IL-13-induced death of activated microglia. A and B, Cells were cotreated for 6 days with the indicated combinations of LPS (10 ng/ml), IL-13 (20 ng/ml), NS398 (10 µM in A and 10 and 20 µM in B), and celecoxib (2.5 and 5 µM in B). At 6 days after the treatment, cell death was determined using calcein-AM and Etd-1. Fluorescence micrographs were obtained using a Nikon Diaphot 300 (A). The number of Etd-1-positive dead cells was counted in eight fields from two different wells. Values represent means ± SEM of eight replicates (B). C, NS398 was added together with LPS and IL-13 (co) or 24, 48, or 72 h after cells were cotreated with LPS and IL-13 (24A, 48A, and 72A, respectively). At 6 days after cells were treated with LPS and IL-13, cell death was assayed. The number of Etd-1-positive dead cells was counted, as in B. Values in B and C are the means ± SEM of eight replicates. The data are representative of more than three independent experiments. *p < 0.01, compared with combination of LPS and IL-13.
found that 11-deoxy PGE1, butaprost, and ciglitazone dose dependently induced microglial death (Fig. 5). However, sulprostone had little effect on microglial death (Fig. 5).

Because EP2 agonists (11-deoxy PGE1 and butaprost) and the PPARγ agonist (ciglitazone) induced microglial death, we examined the effect of the EP2 antagonist (AH6809) and the PPARγ antagonist (GW9662) on microglial death induced by cotreatment with LPS and IL-13. We could not examine EP4 antagonists because they are not yet commercially available. Both AH6809 (5–10 μM) and GW9662 (1–3 μM) protected microglia from death induced by cotreatment with LPS and IL-13 (Fig. 6). These results suggest that EP2 and PPARγ receptors play at least a part in microglial death caused by cotreatment by LPS and IL-13.

**IL-13-enhanced COX-2 expression is mediated by JNK**

To investigate the signaling mechanisms that are involved in the enhancement of COX-2 expression by cotreatment with LPS and IL-13, we examined the phosphorylation of MAPKs because they are important signaling molecules that regulate COX-2 expression (20–22). The LPS-induced activation of three MAPK subtypes was differentially regulated by IL-13. Specifically, IL-13 enhanced LPS-stimulated JNK phosphorylation at all time points (6–72 h), whereas IL-13 had little effect on LPS-stimulated ERK or p38 MAPK phosphorylation (Fig. 7A). IL-13 alone did not activate JNK up to 72 h (Fig. 7A). Contrary to IL-13, however, neither
IL-10 nor TGF-β affected LPS-induced phosphorylation of JNK (Fig. 7B).

To further investigate the involvement of JNK in COX-2 expression, we examined the effect of JNK inhibitor, SP600125. Because JNK also mediates the effects of LPS in macrophages (23), cells were first stimulated with LPS and then treated 3 h later with IL-13 and SP600125 to prevent SP600125 from blocking the initial activation of JNK by LPS. We verified that this delayed treatment enhanced LPS-induced COX-2 expression and that 1 and 5 μM SP600125 dose dependently reduced IL-13-enhanced COX-2 expression and PGE2 and 15d-PGJ2 production (Fig. 8, A and B). Consistent with this finding, SP600125 reduced microglial death induced by LPS and later addition of IL-13 (Fig. 8C). We also examined whether JNK mediated PGE2- and 15d-PGJ2-induced microglial death. We found that SP600125 did not protect cells from PGE2- or 15d-PGJ2-induced cell death (Fig. 8D). These results suggest that IL-13 enhances JNK activation, which in turn enhances COX-2 expression in LPS-treated microglia. However, JNK did not mediate the death processes activated by COX-2 products, PGE2 or PGJ2.

**Discussion**

In a previous study, we reported that activated microglia undergo cell death in the presence of IL-13 in vitro and in vivo (8, 9). The results of the present study showed that IL-13 enhances COX-2 expression in activated microglia. More importantly, the enhanced COX-2 expression was associated with microglial death. IL-4 shares receptors with IL-13 and produced similar effects. However, IL-10 and TGF-β neither enhanced COX-2 expression nor induced death of activated microglia.

MAPKs including ERK, JNK, and p38 are involved in the regulation of COX-2 expression (20–22). The present study showed that LPS-induced JNK phosphorylation is significantly increased in the presence of IL-13. However, neither IL-10 nor TGF-β enhanced LPS-induced JNK phosphorylation. These results provide evidence that JNK mediates IL-13-enhanced COX-2 expression. This hypothesis was further supported by the findings that SP600125 reduced IL-13-enhanced COX-2 expression and microglial death, although SP600125 did not reduce microglial death induced by the exogenous addition of COX-2 products, PGE2 and 15d-PGJ2.

PGE2 and 15d-PGJ2 seemed to induce microglial death through the activation of EP2 and PPARγ, although we could not exclude the involvement of EP4. In the current study, we found that an EP2 agonist (butaprost), an EP2 and EP4 agonist (11-deoxy PGE2), and a PPARγ agonist ( ciglitazone) dose dependently induced microglial death. More importantly, EP2 and PPARγ antagonists reversed microglial death induced by cotreatment with LPS and IL-13. The amount of PGE2 and 15d-PGJ2 produced from microglia cotreated with LPS and IL-13 was in the range of 10–20 nM, and exogenously added PGE2 and 15d-PGJ2 dose dependently induced microglial death at the range of 10 nM to 1 or 5 μM. Furthermore, cotreatment of PGE2 and PGJ2 showed additive cytotoxicity compared with that induced by PGE2 or PGJ2 alone. Therefore, these results support the idea that PGE2 and 15d-PGJ2 participate in IL-13-induced microglial death due to the involvement of EP2 and PPARγ.

A major issue raised by the present study is the physiological consequence of increased expression of COX-2 in the presence of IL-13. COX-2 is generally accepted to be a proinflammatory mediator because several inflammatory stimulators induce the expression of COX-2, and a major COX-2 product, PGE2, induces fever and pain, increases vascular permeability, and recruits inflammatory cells to sites of inflammation (14). More importantly, COX inhibitors have been clinically used to diminish inflammation. However, COX-2 expression itself may not be an indication of an inflammatory state. Recently, several pieces of evidence were reported suggesting that COX-2 is involved in the resolution of inflammation (24–26). Gilroy et al. (27) showed that in the carrageenan-induced pleurisy model, COX-2 expression is up-regulated at two phases, the first at 2 h and the second at 48 h after carrageenan administration. They also showed that COX-2 inhibitors have differential effects on inflammation depending on the times the drugs were administered: early administration suppressed inflammation, but late administration exacerbated inflammation (27). In an agreement with these findings, vanadium pentoxide-induced pulmonary inflammation is accelerated in COX-2-/- mice (28). Furthermore, inhibition of COX-2 increases mucosal inflammation and blocked resolution of inflammatory responses in the acute lung injury (29, 30). Indeed, the cyclopentenone PG product of COX-2, 15d-PGJ2, is generally accepted to be an anti-inflammatory PG. The 15d-PGJ2 reduces the expression of proinflammatory mediators from microglia and astrocytes (13, 31, 32). PGJ2 also enhances the death of Raw264.7 macrophages induced by LPS and IFN-γ.
which is an important mechanism for the resolution of inflammation (33). As observed in this study and as previously reported, 15d-PGJ2, anti-inflammatory roles of PGE2 have been suggested. PGE2 reduces the expression of inducible NO synthase (iNOS) and IL-1β (35, 36). PGE2 increases levels of anti-inflammatory lipid mediators, such as lipoxins (30, 37). In this study, we showed that PGE2 induced cell death in microglia. Interestingly, the cytotoxic effect of COX-2 and PGE2/15d-PGJ2 is cell type specific. Astrocytes are resistant to the PGE2 and 15d-PGJ2 at the concentrations that were cytotoxic to microglia. Cancer cells are also resistant to COX-2 and PGE2 (38). Therefore, inflammatory cells such as microglia and macrophages could be selectively sensitive to PGE2 and/or PGJ2.

Several studies have reported that COX-2 is associated with cytotoxicity in brain diseases (39–43) because nonsteroidal anti-inflammatory drugs (NSAIDs) reduce ischemic damage and the progression of Alzheimer’s and Parkinson’s diseases (39–43). However, the neuroprotective effect of NSAIDs could arise from diverse mechanisms independently from the inhibition of microglial COX-2. One possibility is COX-independent inhibition of NF-κB and AP-1. Because NO (a product of iNOS) and TNF-α have been known to be toxic to neurons (44–46), the protective effect of NSAIDs could be at least partly due to the reduced expression of these cytokotic inflammatory mediators as a result of inhibition of NF-κB and AP-1 (47). Second, some of, but not all NSAIDs directly affect amyloid pathology in the brain by altering γ-secretase activity and reducing β-amyloid peptide levels independently of COX activity (48).

The current results might be irrelevant for proinflammatory actions of COX-2 in microglia. COX-2 inhibitors could produce complex results in brain inflammation. COX-2 inhibitors could inhibit microglial expression of iNOS and TNF-α, but, in contrast, COX-2 inhibitors could prolong inflammatory reactions by inhibiting the death of activated microglia. Therefore, all of these results underscore the need for further thorough studies of COX-2 function and the effects of COX inhibitors in the brain.

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Disclosures

The authors have no financial conflict of interest.

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