Microglial EP2 as a New Target to Increase Amyloid β Phagocytosis and Decrease Amyloid β-Induced Damage to Neurons

Feng-Shiun Shie[1]; Kathleen S. Montine[1]; Richard M. Breyer[2]; Thomas J. Montine[1]

[1] Department of Pathology, University of Washington, Seattle.
[2] Department of Medicine, Vanderbilt University, Nashville, Tenn.

Corresponding author:
Thomas J. Montine, Department of Pathology, University of Washington, Box 359645, Harborview Medical Center, 325 9th Ave, Seattle, WA 98104, USA
(E-mail: tmontine@u.washington.edu)

Epidemiologic and animal model data support a role for the prostaglandin pathway in AD pathogenesis. However, unexpected toxicity from protracted use of some nonsteroidal anti-inflammatory drugs (NSAIDs) compels investigation of therapeutic targets in this pathway other than COX inhibitors. Previously, we have shown that mice lacking one specific receptor for PGE_2, EP2 (EP2-/-), are protected from the indirect neurotoxic effects of cerebral innate immune response mediated by CD14-dependent activation. Here we review data showing that EP2-/- microglia have a highly desirable combination of features: ablated indirect neurotoxicity following exposure to $A\beta_{1-42}$ coupled with enhanced phagocytosis of Aβ peptides, both synthetic and those deposited in human brain. These data point to microglial EP2 as a more focused target within the PG pathway for therapy in AD.

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Pathologic processes of AD are thought to precede clinically diagnosed dementia by as much as 2 or 3 decades. Indeed, as early as 1976, Katzman applied the chronic disease model to AD and proposed the existence of a *latent stage* where some structural damage accrues but there are no functional or behavioral changes, followed by a *prodromal stage* during which more structural damage accrues and mild functional and behavioral changes occur, and ultimately a *clinical stage* with substantial irreversible damage and behavioral abnormalities (14). AD latency and prodrome are receiving increasing attention because it is here that interventions would have the greatest impact and because of a growing realization that treatment strategies for these earlier stages may be different from treatment of established dementia (18).

Large observational studies repeatedly have associated a markedly decreased incidence of AD with use of NSAIDs for more than 2 years (2, 12, 30, 34); indeed, these and other observations studies form a large part of the evidence in support of the proposal that AD derives significantly from innate immune activation in diseased regions of brain. In contrast, a randomized clinical trial that evaluated 2 different NSAIDs failed to show benefit in patients with established AD (1). One interpretation of these results is that while COX inhibition prevents AD by suppressing processes important in latency, inhibiting these mechanisms later in clinical AD is no longer capable of significantly impeding disease progression. Although these associative studies show only partial effectiveness—along with results from transgenic mouse models of cerebral Aβ amyloidogenesis (13, 17, 33)—they provided sufficiently compelling rationale to embark on a large clinical trial to evaluate a non-selective COX inhibitor, naproxen, and a COX-2 selective inhibitor, celecoxib, in the prevention of AD, the Alzheimer Disease Anti-inflammatory Prevention Trial (ADAPT) (18).

NSAIDs are thought to act primarily through inhibition of the cyclooxygenases (COXs), enzymes that catalyze the committed step in prostaglandin (PG) and thromboxane (Tx) synthesis (Figure 1). The immediate product of COX is an unstable intermediate that is efficiently catalyzed to a group of PGs and TxA_2 that exert potent autocrine and paracrine effects via activation of a growing family of G protein-coupled receptors (5). While NSAIDs have other effects in vitro, we are unaware of any study demonstrating that the major therapeutic effect of NSAIDs is mediated by mechanisms other than COX inhibition. For example, the recent proposal from tissue culture experiments that some COX inhibitors may act by alteration of γ-secretase activity (32) has not been supported in vivo (15).

NSAIDs are a chemically diverse group of drugs that can be divided into three groups: aspirin (acetylsalicylic acid or ASA), non-selective COX inhibitors (such as ibuprofen and naproxen), and COX-2-selective inhibitors (such as celecoxib). These agents differ in their clinical use. ASA is most commonly used for thromboembolic prophylaxis because it irreversibly inhibits COX-1 thereby preventing platelet production of TxA_2, while COX inhibitors are less effective for thrombosis prophylaxis because they reversibly inhibit COX-1 as well as suppress the anti-platelet aggregating factor, PGI_2. In contrast, non-selective COX inhibitors have stronger COX-2 suppressing activity than ASA and so are widely used as anti-inflammatory agents. Protracted use of selective COX-2 inhibitors may indeed have the opposite effect of ASA; by suppressing prostaglandin PGI_2 (prostacyclin) but not platelet TxA_2 (COX-1-derived) these drugs appear to promote thrombotic events (11). This is one explanation for the untoward effects recently associated with protracted use of highly selective COX-2 inhibitors and is part of the rationale behind withdrawal of one COX-2 selective inhibitor from the market. These concerns coupled with unexpected toxicity observed with long term use of naproxen, a non-selective COX inhibitor, also formed the rationale for the recent suspension of ADAPT.

The growing concern over toxicity associated with protracted treatment not only with COX-2-selective inhibitors but also naproxen appropriately clouds the potential of these drugs as neuroprotective agents. However, despite these toxicity concerns, the epidemiologic data underpinning ADPAT and the data from transgenic models that support COX inhibition as a means of suppressing Aβ accumulation remain sound. In other words, the PG pathway is still a promising target for the prevention of AD; however, we need to identify pharmacologic targets in this pathway that are more focused than COX inhibition. Indeed, a major shift in research is now occurring with attention turning away from COX inhibition to blockade of specific PG receptors.

PGE_2 is distinct from other eicosanoid products of COX because of the existence of widely expressed multiple receptor subtypes, EP1, EP2, EP3, and EP4, linked to functionally antagonistic second messenger systems; because of this PGE_2 has versatile and often opposing actions in tissues and cells (5). All of the EP receptors are expressed in rodent brain, where there are regional and cell-specific differences in expression and activity (4, 7-9, 23-25, 31, 36). EP4 expression is highly restricted to some hypothalamic nuclei (36). EP3 is predominantly expressed on neurons (8, 9, 24, 31), whereas EP1 and EP2 are expressed on glia and neurons (6, 16). Others have shown that EP2-/- mice are more vulnerable to ischemia-reperfusion injury (19). We have shown that indirect neuronal damage by CD14-dependent innate immune activation in vivo is completely suppressed in 2 different strains of mice that are null for EP2 (20, 21, 35).

While CD14 activation is one mechanism by which Aβ fibrils lead to microglial activation (10), determining the role of microglial activation in our in vivo experiments was limited because EP2 is expressed on multiple cell types in brain. Therefore we pursued the role of microglial EP2 in response to Aβ exposure in cell culture. Our first experiments examined the neurotoxicity of Aβ peptides by exposing primary cultures of neurons or co-cultures of neurons and microglia to aggregated $Aβ_{1-42}$ (Table 1) (28). Similar to the results of several other laboratories, we observed that aggregated $Aβ_{1-42}$ is directly toxic to wt neurons, and

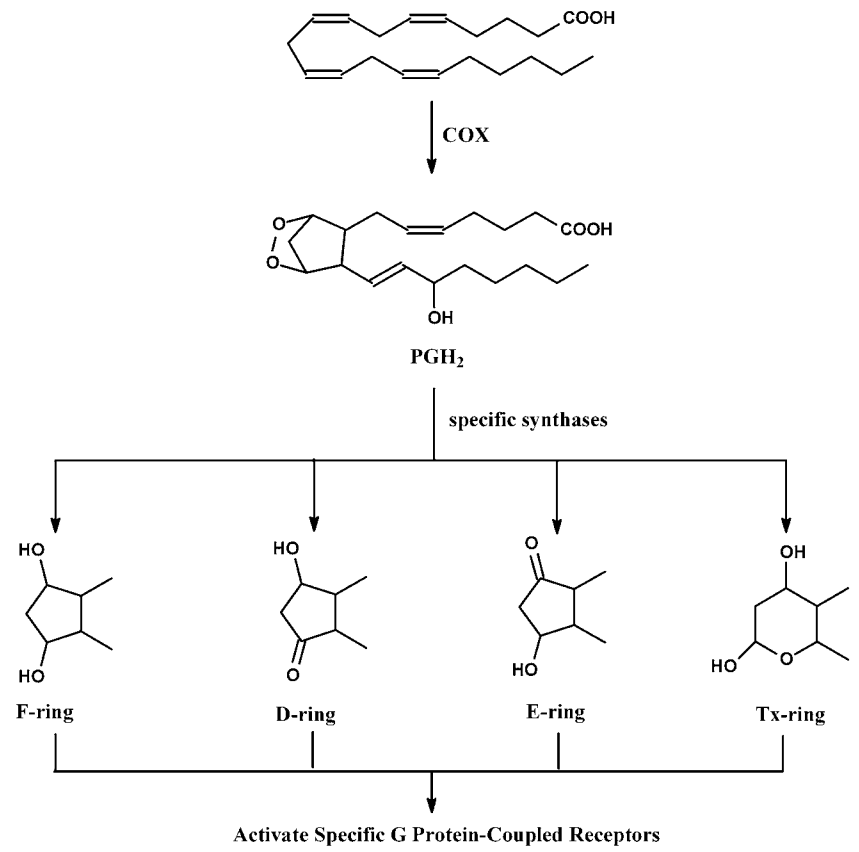

Figure 1. Diagram of eicosanoid products generated from COX and their receptors.

Neuron	wt	wt	wt	wt
Microglia	none	wt	wt	EP2-/-
$Aβ_{1-42}$	+	+	+	+
IBU	none	none	+	none
% Change in TUNEL + Neurons	196 ± 10	259 ± 13	109 ± 8	59 ± 5

Table1. *Suppression of neurotoxicity from $Aβ_{1-42}$ by ibuprofen or ablation of microglial EP2.* Co-cultures of primary mouse cerebral neurons and microglia were exposed to aggregated synthetic $Aβ_{1-42}$ and then assayed for neurotoxicity by counting MAP-2-immunoractive (IR) cells (neurons) that also were positive for TUNEL staining. Data are expressed as % of wt neurons not exposed to $Aβ_{1-42}$.

the neurotoxicity of $Aβ_{1-42}$ is significantly further increased in the presence of wt microglia (26). Moreover, direct and indirect neurotoxicity of $Aβ_{1-42}$ can be completely suppressed by NSAIDs such as ibuprofen (IBU). These last results are difficult to interpret precisely because COX inhibitors are active in both neurons and microglia in co-cultures. However, genetically modified microglia can help resolve these issues. Indeed, in sharp contrast to wt microglia, when wt neurons co-cultured with EP2-/- microglia were exposed to aggregated $Aβ_{1-42}$ there was no increase in neurotoxicity; in fact, neuronal damage was slightly but significantly lower than control in the presence of EP2-/- microglia. These data show that unlike wild type microglia, EP2-/- microglia do not mount a neurotoxic response following exposure to $Aβ_{1-42}$ and may even mildly protect neurons from the direct toxic effects of $Aβ_{1-42}$.

Several potential effectors of innate immunity mediated neuronal damage have been proposed and include products of several enzymes, principal candidates being inducible nitric oxide synthase (iNOS) and COX-2, either alone or in combination. We

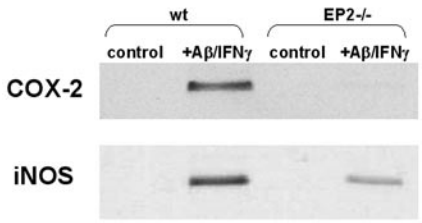

Figure 2. Primary cultures of mouse cerebral microglia were untreated or exposed to 12 μM $A\beta_{1-42}$ plus 10 ng/ml interferon-γ (required for Aβ-mediated activation of microglia in culture) for 24 hr and then solubilized and analyzed by Western blot for COX-2 or iNOS.

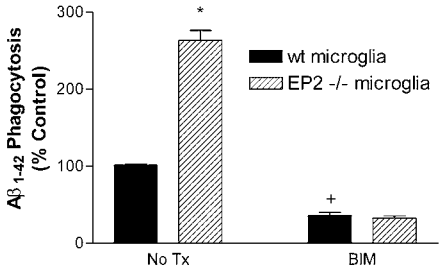

Figure 3. Primary cultures of mouse cerebral microglia were incubated fluoroscein-labeled $A\beta_{1-42}$ with and without BIM (10 μM), a PKC inhibitor. Data are % of wt microglia incubated with fluoroscein-labeled $A\beta_{1-42}$ only. Two-way ANOVA had P<0.0001 microglia genotype, presence or absence of BIM, and interaction between these terms. Bonferroni-corrected posttests had *P<0.0001 for wt versus EP2-/- without BIM, but P>0.05 for cultures with BIM. Wt microglia with and without BIM had $^{+}$P<0.01.

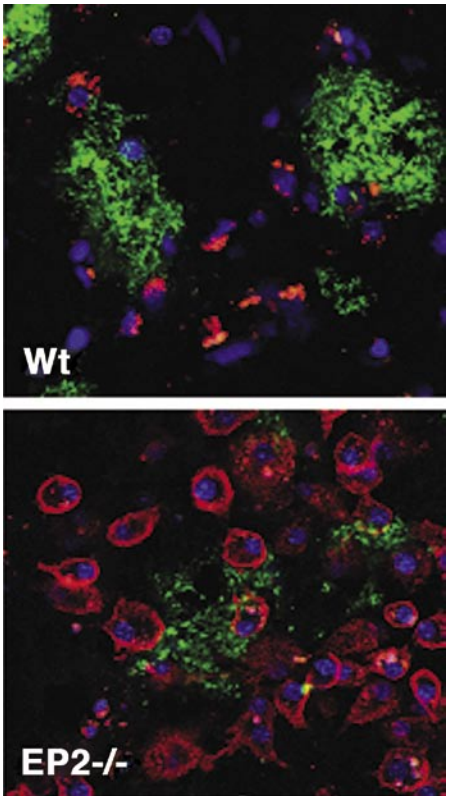

Figure 4. Aβ phagocytosis was evaluated ex vivo by incubating primary cultures of mouse cerebral microglia with consecutive cryosections of hippocampus from a patient who died of AD (×250). Compared to wt, EP2-/- microglia showed decrease tissue Aβ immunoreactivity (green) and increase in one marker of microglial activation, CD11b (red). Nuclei are stained blue.

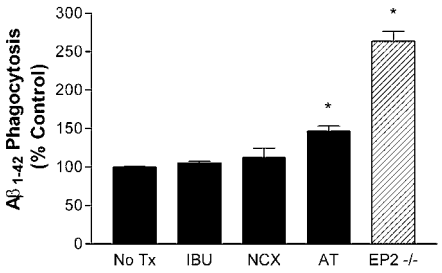

Figure 5. Primary cultures of mouse cerebral microglia were exposed to drug for 24 hours and then to drug plus fluoroscein-labeled $A\beta_{1-42}$ (100 nM) for another 24 hours prior to measurement of fluoroscein uptake by flow cytometry. Data are % of wt microglia incubated with fluoroscein-labeled $A\beta_{1-42}$ only. Solid bars are for results from wt microglia incubated with no drug, ibuprofen (IBU, 10 μM), NCX (NCX, 1 μM), or a-tocopherol (AT, 10 μM). Striped bar is for EP2-/- microglia with no drug added. One-way ANOVA had P<0.0001 with Bonferroni-corrected repeated paired comparisons having *P<0.001 for wt/AT or EP2-/- versus wt/no treatment.

examined these proposed sources of neurotoxic products in wt and EP2-/- microglia following activation by aggregated $A\beta_{1-42}$. As seen in the Western blots in Figure 2, microglia from EP2-/- mice had significantly less induction of iNOS and COX-2 following incubation with $A\beta_{1-42}$ (28).

While the precise mechanisms by which EP2-mediated signaling contributes to increased levels of iNOS and COX-2 are not clear from these experiments, others have demonstrated the capacity of PGE_2 to modulate expression or activity of both of these enzymes (22). Nevertheless, our results do clearly show that microglia specifically lacking EP2 do not cause neurotoxicity in vitro following activation by $A\beta_{1-42}$, likely related to diminished induction or activation of enzymes that catalyze the formation of known neurotoxic products. It is noteworthy that these results are in complete agreement with our in vivo studies where we have specifically stimulated CD14-dependent innate immune response in microglia and shown ablation of neurotoxicity in EP2-/- mice (20, 21, 35). In combination, these data provide a mechanistic rationale for the observed apparent protective effects of NSAIDs like IBU in decreasing the risk for developing AD. Most exciting is that they appear to replicate the effect of COX inhibitors while focused on a much more specific pharmacologic target, EP2.

Our cell culture data suggested that EP2-/- microglia might protect neurons from the direct toxic effects of $A\beta_{1-42}$. One possible mechanism for this could be increased Aβ phagocytosis by EP2-/- microglia. Therefore, we quantified microglial phagocytosis of fluorescein-labeled aggregated $A\beta_{1-42}$ in a cell cytometric assay that measures cellular incorporation of label (Figure 3). Our data showed that EP2-/- microglia had greatly increased fluorescein uptake. Moreover, our data showed that bisindolylmaleimide (BIM), an inhibitor of protein kinase C (PKC), partially suppressed wt microglial uptake of label and returned EP2-/- microglia to wt levels; this last finding strongly suggests that post-receptor signaling cascades are intact in EP2-/- microglia. These results show that EP2-/- microglia have substantially increased phagocytic activity for aggregated $A\beta_{1-42}$ compared to wt microglia.

We tested the hypothesis that EP2-/- microglia had enhanced phagocytosis for Aβ peptides by adapting the assay used by others to test the feasibility of Aβ vaccination by measuring the clearance of opsonized Aβ peptides from sections of human brain (3, 27, 29). It needs to be stressed that no antibodies were used in our experiments to opsonize Aβ or any other peptide; rather tissue sections from hippocampi of patients who died of AD were simply incubated with wt or EP2-/- microglia (Figure 4). In agreement with the work of others who showed that wt microglia do not detectably reduce Aβ peptide tissue burden without opsonization, our wt microglia did not significantly reduce Aβ-immunoreactive plaque material compared to tissue sections not incubated with microglia. In striking contrast, tissue incubated with EP2-/- microglia showed marked reduction in Aβ-immunoreactive plaque material as well as increased microglial CD11b immunoreactivity, a feature of microglial activation. Extraction of multiple tissue samples following incubation with either wt or EP2-/- microglia followed by Western blots for Aβ peptides showed an approximately 50% reduction in both $A\beta_{1-40}$ and $A\beta_{1-42}$ in tissue incubated with EP2-/- microglia compared to tissue incubated with wt microglia (28).

Finally, we sought to determine if IBU, or another NSAID, NCX-2216 (NCX), a nitric oxide-releasing flurbiprofen derivative, could mimic the effects of EP2-/- with respect to $A\beta_{1-42}$ phagocytosis (Figure 5). Neither NSAID at concentrations achieved by free drug in plasma altered $A\beta_{1-42}$ phagocytosis by wt microglia. These results show that these two NSAIDs, which have the effect of suppressing all eicosanoid products derived from COX, have no net effect on $A\beta_{1-42}$ phagocytosis, yet specific interruption of signaling by one PGE2 receptor greatly enhances $A\beta_{1-42}$ phagocytosis. As a positive control, we observed that α-tocopherol (AT), at concentrations achieved in plasma with dietary supplementation, did modestly increase $A\beta_{1-42}$ phagocytosis by wt microglia. However, this increase in microglia $A\beta_{1-42}$ phagocytosis was small compared to the increase achieved by specifically ablating EP2.

In conclusion, while epidemiologic and animal model data support a role for the PG pathway in AD pathogenesis, the unexpected toxicity from protracted use of NSAIDs compels investigation of specific therapeutic targets in this pathway other than COX inhibition. Previously, we have shown that EP2-/- mice are protected from the indirect neurotoxic effects of cerebral innate immune response mediated by CD14-dependent activation. Here, we reviewed data showing that microglia lacking EP2 have a highly desirable combination of features: ablated indirect neurotoxicity following exposure to $A\beta_{1-42}$ and enhanced phagocytosis of Aβ peptides, both synthetic and those deposited in human brain. Together, these data point to microglial EP2 as a more focused target for therapy in AD.

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