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Deletion of the Prostaglandin E_2 EP2 Receptor Reduces Oxidative Damage and Amyloid Burden in a Model of Alzheimer's Disease

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Epidemiological studies demonstrate that chronic use of nonsteroidal anti-inflammatory drugs (NSAIDs) in normal aging populations reduces the risk of developing Alzheimer's disease (AD). NSAIDs inhibit the enzymatic activity of cyclooxygenase-1 (COX-1) and inducible COX-2, which catalyze the first committed step in the synthesis of prostaglandins. These studies implicate COX-mediated inflammation as an early and potentially reversible preclinical event; however, the mechanism by which COX activity promotes development of AD has not been determined. Recent studies implicate the prostaglandin E_2 (PGE_2) E prostanoid subtype 2 (EP2) receptor in the development of the innate immune response in brain. Here, we report that deletion of the PGE_2 EP2 receptor in the APPSwe-PS1ΔE9 model of familial AD results in marked reductions in lipid peroxidation in aging mice. This reduction in oxidative stress is associated with significant decreases in levels of amyloid-β (Aβ) 40 and 42 peptides and amyloid deposition. Aged APPSwe-PS1ΔE9 mice lacking the EP2 receptor harbor lower levels of β C-terminal fragments, the product of β-site APP cleaving enzyme (BACE1) processing of amyloid precursor protein. Increases in BACE1 processing have been demonstrated in models of aging and AD and after oxidative stress. Our results indicate that PGE_2 signaling via the EP2 receptor promotes age-dependent oxidative damage and increased Aβ peptide burden in this model of AD, possibly via effects on BACE1 activity. Our findings identify EP2 receptor signaling as a novel proinflammatory and proamyloidogenic pathway in this model of AD, and suggest a rationale for development of therapeutics targeting the EP2 receptor in neuroinflammatory diseases such as AD.

Key words: **prostaglandin; receptor; transgenic; inflammation; Aβ peptide; Alzheimer's disease**

Introduction

Changes in demographics resulting in larger aging populations will cause the incidence of Alzheimer's disease (AD) to increase markedly over the next several decades. The understanding of early preclinical events and the development of preventive strategies will be critical in the management of this disease. Of relevance to early events in AD is the overwhelming evidence for a central role of inflammation and oxidative stress (Akiyama et al., 2000). Epidemiological data support a preventive as opposed to a therapeutic effect of COX inhibition in AD, in which nonsteroidal anti-inflammatory drug (NSAID) administration delays the onset and risk of developing AD (Breitner et al., 1995; McGeer et al., 1996; Stewart et al., 1997; in t' Veld et al., 2001; Szekely et al., 2004). This preventive effect has been modeled in transgenic mice expressing mutant forms of amyloid precursor protein (APP) and presenilin 1 (PS1), and, in these models, NSAIDs significantly reduce amyloid deposition (Lim et al., 2000; Jantzen et al., 2002; Yan et al., 2003) and microglial activation (Lim et al., 2000; Yan et al., 2003). NSAIDs inhibit the activity of the constitutive cyclooxygenase 1 (COX-1) and inducible COX-2 enzymes, which catalyze the formation of prostaglandin H_2 (PGH_2) from arachidonic acid, the precursor of the five prostaglandins: PGE_2, PGD_2, PGI_2, $PGF_{2\alpha}$, and thromboxane A_2 (TXA_2). Significantly, levels of PGE_2, a principal proinflammatory product of COX enzymatic activity, have been found to be elevated in patients with probable AD (Montine et al., 1999), suggesting that PGE_2 signaling may function in the development of AD.

PGE_2 binds to four G-protein-coupled receptors designated E-Prostanoid 1-4 (EP1-4) that have divergent effects on cAMP production and phosphoinositol turnover (Breyer et al., 2001). The PGE_2 EP2 receptor is highly expressed in hippocampus and cerebral cortex (McCullough et al., 2004), structures that are significantly affected in AD. Importantly, recent studies have demonstrated that the EP2 receptor functions in the microglial innate immune response to the bacterial endotoxin lipopolysaccaride

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(LPS) and promotes the formation of reactive oxygen species; after administration of LPS, EP2−/− mice fail to mount the inflammatory oxidative response seen in EP2+/+ mice, as assayed by levels of lipid peroxidation (T. J. Montine et al., 2002). The amyloid hypothesis of AD suggests that dysregulation of amyloid-β (Aβ) peptide metabolism triggers the innate immune response, in which microglial cells are activated to clear and phagocytose Aβ, producing free radical species and neurotoxic cytokines. The CD14-dependent innate immune response to LPS is directly relevant to the immune response to accumulating Aβ peptide, in which microglial activation, phagocytosis, and elaboration of inflammatory mediators are, in part, CD14 dependent (Fassbender et al., 2004; Milatovic et al., 2004). Given that Aβ accumulation can elicit an innate immune response similar to that of LPS, we hypothesized that the EP2 receptor functioned similarly in mediating inflammatory oxidative damage in a transgenic model of familial AD. Given the association between increased oxidative stress and Aβ accumulation (Pratico et al., 2001), we tested the hypothesis that the proinflammatory effects of PGE_2 signaling via the EP2 receptor played a role in the development of pathology in the APPSwe-PS1ΔE9 model of familial AD (Jankowsky et al., 2001).

Materials and Methods

Animals. This study was conducted in accordance with the National Institutes of Health guidelines for the use of experimental animals. Protocols were approved by the Institutional Animal Care and Use Committee at Johns Hopkins University. C3HeJ × C57BL/6J APPSwe-PS1ΔE9 mice were kindly provided by Dr. D. Borchelt (Department of Pathology, Johns Hopkins University, Baltimore, MD). APPSwe-PS1ΔE9 ($n = 2–4$ backcrosses to C57BL/6J) were used as breeders and crossed to C57BL/6J EP2−/− mice to generate APPSwe-PS1ΔE9:EP2+/− mice; these were crossed to C57BL/6J EP2−/− mice to generate APPSwe-PS1ΔE9:EP2−/− mice. Parallel crosses were made between APPSwe-PS1ΔE9 and C57BL/6J mice to generate cohorts of APPSwe-PS1ΔE9:EP2+/+ control mice. The APPSwe transgene encodes a mouse–human hybrid transgene containing the mouse sequence in the extracellular and intracellular regions, and a human sequence within the Aβ domain with Swedish mutations K594N/M595L. The PS1ΔE9 transgene encodes the exon-9-deleted human presenilin-1. Both transgenes are coexpressed under the control of the mouse prion promoter with plaque deposition beginning at 5–6 months (Jankowsky et al., 2001). Cohorts of 7–11 female or male APPSwe-PS1ΔE9 mice in EP2+/+ and EP2−/− backgrounds were allowed to age to 8 months of age for females and 12 months of age for males. Two distinct time points (8 months in females, and 12 months in males) were used to optimize the chance of identifying an EP2-dependent effect on development of pathology.

Measurement of F2-isoprostanes and F4-neuroprostanes. F2-isoprostanes and F4-neuroprostanes were measured by gas chromatography with negative ion chemical ionization mass spectrometry as described previously (Musiek et al., 2004).

Aβ peptide quantification by ELISA. Flash-frozen brain hemispheres were homogenized in 10 vol of 5.0 M guanidine-HCl, 50 mM Tris, pH 8.0. Homogenates were mixed at room temperature for 4 h, spun at 5000 rpm for 2 min, and supernatants were collected and stored at −80°C. Aβ levels were assayed by sandwich ELISA following the protocol of Mehta et al. (2000) and normalized to total protein concentration (BCA Protein Assay kit; Pierce, Rockford, IL). For Aβ 40 and 42 in aged cohorts, samples were diluted 1:800 and 1:2000 for ELISA, respectively, in PBS, 5% BSA, and 0.03% Tween 20, pH 7.4, supplemented with Complete Protease Inhibitor (Roche Diagnostics, Mannheim, Germany) and phenylmethanesulfonylfluoride (PMSF; Sigma, Allentown, PA). In young preplaque mice (2-month-old female; $n = 4–6$ pairs), brains were homogenized in PBS, pH 7.4, supplemented with protease inhibitors, and Aβ 40 and 42 were assayed at 1:5 and 1:50 dilutions. Levels of Aβ 40 and 42 were measured using the capture monoclonal antibody 6E10 (recognizes N-terminal 1–16 sequence of Aβ peptide; Signet Laboratories, Dedham, MA) and detection of biotinylated anti-Aβ 40 or anti-Aβ 42 antibodies (Signet Laboratories). Sample sizes of aged cohorts ranged from 7 to 11 per gender per genotype, and experiments were performed in duplicate. For quantification of endogenous mouse Aβ 40, monoclonal 4G8 was used as the capture antibody (Signet Laboratories).

Congo red staining and quantification of fluorescence. To quantify β-pleated sheet amyloid deposition, sagittal paraffin sections of brain hemispheres were prepared and every 10th section was stained for Congo red and counterstained with aniline blue. Congo red fluorescence was visualized using a Nikon (Tokyo, Japan) Eclipse E600 microscope connected to an Orca-100 CCD camera (Hamamatsu Photonics, Shizuoka, Japan) and digitized using Openlab (Improvision, Lexington, MA) on a Macintosh G4 (Apple Computers, Cupertino, CA). The hippocampal area in each section was visualized at 40× and outlined. This region of interest (ROI) was imaged and digitized at 12 bits/pixel grayscale using a constant exposure time, offset, and gain for all sections. Fluorescence within each ROI was quantified using density slice software with constant settings for minimum and maximum intensities. Percentage area of fluorescence occupied by congophilic signal was calculated by dividing the area of fluorescence by the total area in the ROI. For each gender and genotype, between 7 and 11 mice were examined, using between 7 and 8 slides per brain.

Stereological point counting of Aβ deposition. Quantification of stereologic point counting of 6E10 immunostained sections from cohorts of 8-month-old females was performed using every 10th paraffin section through the hippocampus. Sections were immunostained for human Aβ peptide using monoclonal antibody 6E10 ($n = 8–12$ sections per brain). Stereological point counting of 6E10 immunoreactivity (Jantzen et al., 2002) of hippocampus was performed using Stereo Imager 5.0 (MicroBrightField, Williston, VT). Regions of hippocampus were selected using the unbiased sampling method (West et al., 1991). A standard group of 20 points in a counting area (measuring 100 × 80 μm) was placed in a systematic random position at 400 μm intervals and examined for 6E10 immunoreactivity. The sum of the points falling over areas positively stained with 6E10 was divided by the total number of grid points sampled to estimate the 6E10-immunopositive area fraction.

Western blot analysis. Brain protein (20 μg) was fractionated by SDS-PAGE and electrophoretically transferred to PVDF membranes (Bio-Rad, Hercules, CA). Blots were probed for Aβ peptide using monoclonal 6E10 (Signet Laboratories) probed for APP using polyclonal N-terminal and for APP and C-terminal fragments (CTFs) using C-terminal anti-APP antibodies (anti-APP N-terminal A8967; C-terminal A8717; Sigma, St. Louis, MO), for PS1ΔE9 using polyclonal anti-N-terminal PS1 antibody (presenilin 1 H-70 or SC-7860 from Santa Cruz Biotechnology, Santa Cruz, CA), for mouse prion protein using polyclonal anti-mouse prion protein (SAF-32; Cayman Chemical, Ann Arbor, MI), for actin using anti-actin antibody (Sigma), for anti-β-tubulin (Promega, Madison, WI), for β-site APP cleaving enzyme (BACE1) using a polyclonal anti-BACE (kind gift from Dr. Phil Wong, Johns Hopkins University, Baltimore, MD). APPSwe-PS1ΔE9/BACE−/− and APPSwe-PS1ΔE9 brain lysates derived from 24-month-old male mice were kindly provided by Dr. Phil Wong as controls for Western blot analysis of β-CTFs. Immunoreactivity was detected using either sheep anti-rabbit or anti-mouse HRP-conjugated secondary antibody (Amersham Biosciences, Arlington Heights, IL), followed by enhanced chemiluminescence (Pierce). Autoradiographic signals were quantified using NIH Image. All experiments were confirmed in triplicate.

Stimulation of APPSwe-PS1ΔE9 cortical neurons and APP75 and APP751Swe stably expressing Chinese hamster ovary cells. Primary cerebral cortical cultures were generated from embryonic day 17 (E17) embryos derived from time-pregnant crosses of APPSwe-PS1ΔE9 mice; neurons were plated at 5×10^5 cells/ml as described previously (McCullough et al., 2004); each embryo was cultured separately, and genotypes were determined by PCR. APP751 and APP751Swe Chinese hamster ovary (CHO) cells (kind gift from Dr. E. Koo) were maintained in DMEM with 10% FBS, penicillin/streptomycin, and 0.2 mg of G418. Cells were plated at 50,000 cells/35 mm well in 6-well plates. Neurons were stimulated at 8 d *in vitro*, and CHO cells were stimulated the day after plating with butaprost (10 nM to 10 μM) or vehicle (0.01% ethanol) for 72 and 48 h,

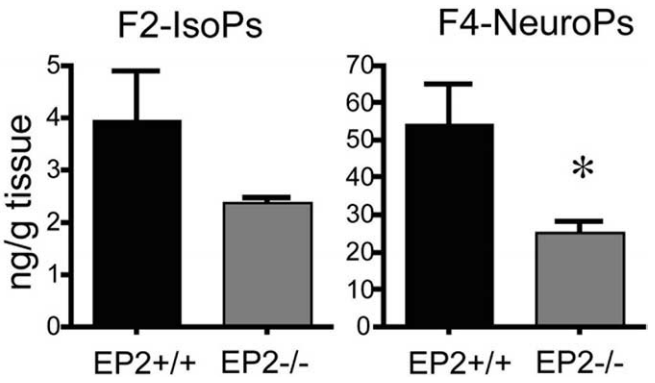

Figure 1. Deletion of the EP2 receptor leads to decreases in lipid peroxidation in APPSwe-PS1ΔE9 mice. Twelve-month-old APPSwe-PS1ΔE9 mice were examined for levels of lipid peroxidation by assaying for F2-isoprostanes (F2-IsoPs), which are free radical-generated isomers of prostaglandin $PGF_{2\alpha}$ in neuronal and non-neuronal cells, and F4-neuroprostanes (F4-NeuroPs), which are neuron-specific products of docosohexanoic acid oxidation. Deletion of the EP2 receptor resulted in decreases in both indices of lipid peroxidation with significance achieved for F4-neuroprostanes ($n = 4$ EP2−/− and 5 EP2+/+; $^*p < 0.05$). Previous studies have demonstrated that unstimulated EP2 wild-type and EP2 null mice do not differ in brain levels of lipid peroxidation (T. J. Montine et al., 2002). Error bars represent SEM.

respectively. After stimulation, culture media were spun at $1000 \times g$ for 5 min, and protease inhibitor mixture (Roche Complete Protease Inhibitor) with 1 μM PMSF was added. Samples were snap frozen at −80°C for subsequent ELISA for Aβ 40 and 42 levels.

Statistical analysis. Student's *t* test or one-way ANOVAs were used. Newman–Keuls *post hoc* test was applied to significant main effects and interactions to estimate differences between particular sets of means. All data are plotted as mean ± SEM.

Results

Deletion of the EP2 receptor in APPSwe-PS1ΔE9 mice reduces levels of oxidative damage

To test the role of PGE_2 signaling through the EP2 receptor in this model of AD, we generated aged cohorts of APPSwe-PS1ΔE9 mice and APPSwe-PS1ΔE9 mice lacking the EP2 receptor. Previous studies of EP2-mediated inflammation in brain have assayed lipid peroxidation as a marker of the inflammatory oxidative response (Montine et al., 1999; T. J. Montine et al., 2002). Because of the significant lipid content of the brain, a sensitive measure of oxidative damage is the level of lipid peroxidation. Accordingly, we measured F2-isoprostanes, which are free radical-generated isomers of prostaglandin $PGF_{2\alpha}$, and F4-neuroprostanes, which are products of docosohexanoic acid oxidation (Musiek et al., 2004) in 12-month-old male cohorts of APPSwe-PS1ΔE9 mice in EP2+/+ and −/− backgrounds. Both indices of lipid peroxidation were reduced in APPSwe-PS1ΔE9 mice lacking the EP2 receptor, with a significant effect observed for the neuron-specific F4-neuroprostanes (Fig. 1) ($p < 0.05$; $n = 4$ EP2−/− and $n = 5$ EP2+/+ backgrounds). Previous studies have established that there are no differences in resting levels of F2-isoprostanes and F4-neuroprostanes in EP2 wild-type and null mice (T. J. Montine et al., 2002), indicating that the EP2 receptor promotes the oxidative response in settings of immune activation. These findings indicate that, in APPSwe-PS1ΔE9 mice, as in the LPS CD14-mediated model of innate immunity, the EP2 receptor promotes an increase in lipid peroxidation.

Deletion of the EP2 receptor in APPSwe-PS1ΔE9 mice reduces levels of Aβ peptides

Oxidative damage has been documented in transgenic models of amyloidosis, in which it is associated with increased levels of Aβ peptides. Accordingly, because of the increases in oxidative stress seen in the EP2+/+ background, we examined cohorts of 8-month-old female and 12-month-old male APPSwe-PS1ΔE9 mice in EP2+/+ and −/− backgrounds for levels of Aβ peptides and plaque load. APPSwe-PS1ΔE9 mice develop amyloid deposits beginning at 5–6 months of age. Deletion of the EP2 receptor resulted in a significant decrease in females and males of both Aβ 40 and 42 levels by ELISA of total guanidine-extracted Aβ peptides (Fig. 2*A*). Similar decreases in levels of Aβ peptide were observed using quantitative Western blot analysis of Aβ peptide levels in EP2−/− compared with EP2+/+ backgrounds (Fig. 2*B*,*C*). Examination of transgenic protein levels of APP and mutant PS1ΔE9 normalized to actin did not reveal differences between genotypes (Fig. 2*D*,*E*). Levels of endogenous mouse prion protein also did not differ between genotypes, confirming that EP2 receptor signaling did not impact on transcription off the transgenic mouse prion protein promoter (Fig. 2*E*).

To quantify amounts of deposited β-amyloid, dense core β-pleated sheet and total amyloid were assayed using Congo red staining and stereological point counting of 6E10 immunostained tissue, respectively (Fig. 3). Amyloid plaque load was examined in the hippocampus, an area that is significantly impacted by amyloid deposition in AD and in transgenic models of mutant APP and PS1. Quantitative analysis of Congo red fluorescence demonstrated a significant reduction in accumulated β-pleated sheet in the EP2−/− background compared with the EP2+/+ background in both 8-month-old female and 12-month-old male cohorts (Fig. 3*A*,*B*). Additional confirmation was performed in 8-month-old females with stereological point counting of 6E10 immunostained tissue. The antibody 6E10 is specific to the first 1–17 amino acids of Aβ 40 and 42, and stereological quantification of immunostained Aβ has been successfully used to quantify plaque load in a model of familial AD treated with NSAIDs (Jantzen et al., 2002). There was a significant decrease in total core and noncore deposited Aβ in the EP2−/− background (Fig. 3*C*,*D*), consistent with the decreases in total Aβ 40 and 42 by ELISA.

Deletion of the EP2 receptor in APPSwe-PS1ΔE9 mice reduces levels of β-CTF

APP is processed first by the β-secretase BACE1 to form the β-CTF, which is subsequently cleaved by the γ-secretase complex to form Aβ 40 or 42 peptides. We examined the processing of APP to β-CTF by BACE1 and tested the possibility that reduced processing by BACE1 might be occurring in the EP2−/− background and resulting in parallel decreases in Aβ 40 and 42 peptides. We first examined levels of BACE1 protein by quantitative Western blot analysis and did not find significant differences between EP2 backgrounds (Fig. 4*A*). We then measured BACE1 processing by quantifying levels of its product, β-CTF, in cohorts of aged APPSwe-PS1ΔE9 mice in the EP2+/+ and −/− backgrounds (Fig. 4*B*,*C*). The identity of the β-CTF fragment was confirmed by examining brain lysates derived from aged APPSwe-PS1ΔE9 mice (24 months of age) lacking BACE1 (Fig. 4*B*, AP-PS/BACE1−/−, second lane). APPSwe-PS1ΔE9 mice lacking the EP2 receptor demonstrated a significant decrease in levels of β-CTF in both female and male cohorts. Levels of α-CTF by quantitative Western blot analysis do not show significant differences by densitometry between EP2 backgrounds (female EP2+/+ vs EP2−/− background, $p = 0.35$; male EP2+/+ vs EP2−/− background, $p = 0.66$; $n = 4$ pairs/genotype). Thus, in aged APPSwe-PS1ΔE9 mice lacking EP2 receptor, β-CTF precursor and Aβ peptide levels were significantly decreased, suggesting

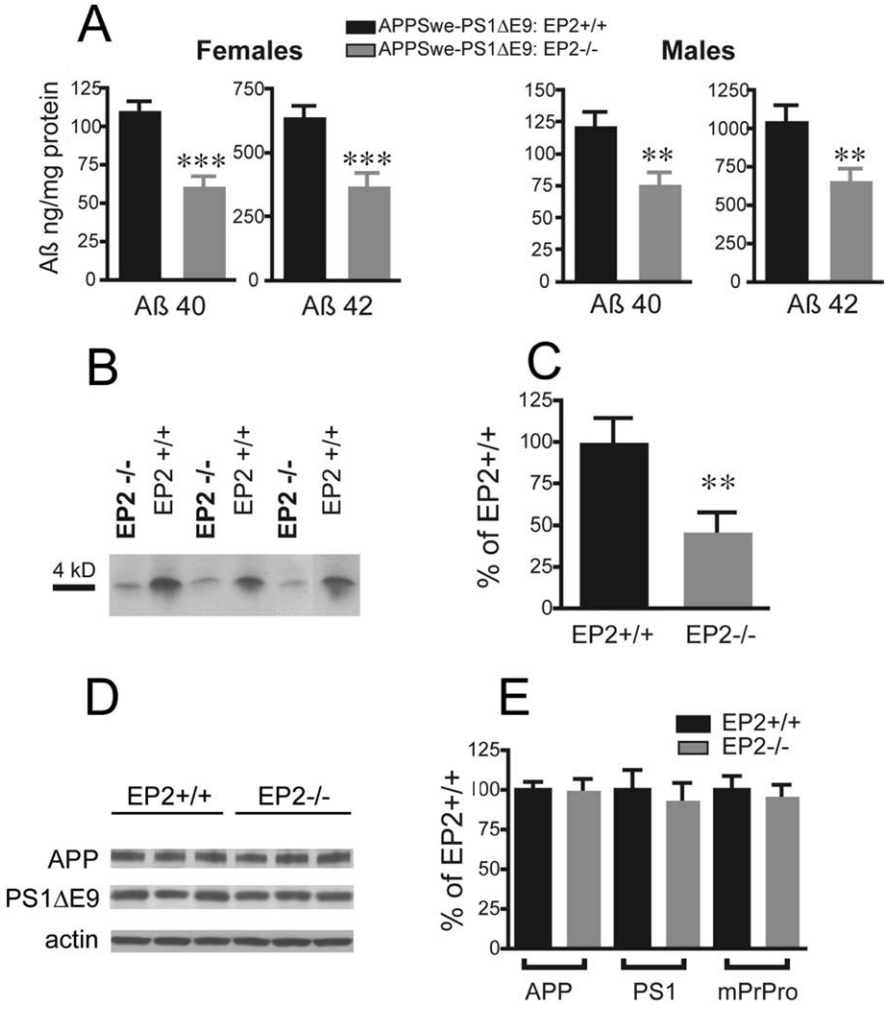

Figure 2. Levels of total Aβ 40 and 42 are reduced in APPSwe-PS1ΔE9 mice lacking the PGE_2 EP2 receptor. ***A***, Total guanidine-extracted Aβ 40 and 42 levels are significantly decreased with EP2 receptor deletion (***$p < 0.001$; $n = 9$ and 6 female mice in EP2+/+ and −/− backgrounds, respectively; **$p < 0.01$, $n = 11$ and 7 male mice in EP2+/+ and −/− backgrounds, respectively). ***B***, Representative Western blot analysis of APPSwe-PS1ΔE9 brain lysates in EP2+/+ and EP2−/− backgrounds using 6E10 antibody. ***C***, Quantification of densitometry demonstrates reduced Aβ in EP2−/− background (**$p < 0.01$; $n =$ 4–5 pairs of females). ***D***, Quantitative Western blot analyses of transgenic APP/APPSwe and PS1ΔE9 proteins do not show differences between genotypes [anti-N-terminal APP polyclonal antibody; similar results (data not shown) were obtained with anti-C-terminal APP polyclonal antibody]; the PS1ΔE9 results in a novel band at 28 kDa (anti N-terminal PS1 antibody), which is not present in nontransgenic brain (data not shown). ***E***, Densitometry of bands for APP, PS1ΔE9, and endogenous mouse prion protein (mPrPro) normalized to actin did not show differences between genotypes ($n = 4$–5 pairs of females). Mouse PrPro was assayed to rule out changes in transgenic mPrPro promoter activity. Error bars represent SEM.

that the EP2 receptor may modulate BACE1 processing of APP and Aβ peptide generation.

Association of oxidative inflammatory response and increased Aβ peptide levels in APPSwe-PS1ΔE9 in EP2+/+ background

Recent studies have demonstrated that BACE1 processing of APP is increased in aging and AD (Fukumoto et al., 2002, 2004; Holsinger et al., 2002; Yang et al., 2003; Li et al., 2004), and this may relate to effects of increased oxidative stress on BACE1 activity (Tamagno et al., 2002; Apelt et al., 2004). EP2 receptor signaling leads to a marked inflammatory oxidative response in the LPS model of innate immunity (T. J. Montine et al., 2002) and in the present model in aged APPSwe-PS1ΔE9 mice (Fig. 1). To test whether the EP2 receptor directly modulates Aβ peptide levels independent of increased oxidative stress, we examined levels of endogenous murine Aβ 40 in EP2 wild-type and null mice, which do not show differences in levels of oxidative stress under basal conditions (T. J. Montine et al., 2002). There were no differences in levels of Aβ 40 in either the wild-type or null mutant EP2 mice (Fig. 4*D*). Examination of a direct effect of EP2 signaling was also performed *in vitro* in primary cortical neurons derived from E17 APPSwe-PS1ΔE9 embryos and in CHO cells stably expressing either hAPP751 or hAPP751Swe protein. Pharmacologic stimulation with the selective EP2 receptor agonist butaprost (Gardiner, 1986) ($EC_{50} = 23$ nM) did not alter production of Aβ42 in APPSwe-PS1ΔE9 neurons (Fig. 4E) or Aβ 40 or 42 levels in hAPP751 or hAPP751Swe CHO cells (Fig. 4F).

We then tested whether young APPSwe-PS1ΔE9 mice at an age before the onset of plaque deposition would have similar changes in levels of Aβ peptides and β-CTF. Two-month-old APPSwe-PS1ΔE9 mice in EP2+/+ and EP2−/− backgrounds were examined (Fig. 4*G–I*). At this age, there were no differences in levels F2-isoprostanes or F4-neuroprostanes in EP2+/+ or −/− backgrounds (Fig. 4G), in contrast to older mice, as illustrated in Figure 1. There were also no associated differences in levels of Aβ 40 and 42 (Fig. 4*H*) or levels of β-CTF (Fig. 4*I*) in EP2+/+ or −/− backgrounds. Thus, in the absence of increased oxidation, no differences were detected in levels of Aβ peptides or β-CTFs in the EP2−/− background. Together, these data suggest an indirect and age-dependent effect of the EP2 receptor on the oxidative response and levels of β-CTF and Aβ peptides.

Discussion

The present study demonstrates a novel proinflammatory and proamyloidogenic function for the PGE_2 EP2 receptor in a model of familial AD. The APPSwe-PS1ΔE9 model of amyloidosis is characterized by a marked increase in Aβ42/40 ratios associated with inflammatory and behavioral changes (Borchelt et al., 1997; Jankowsky et al., 2004; Savonenko et al., 2005). In this model, deletion of the EP2 receptor results in marked decreases in lipid peroxidation in aged mice that are associated with significantly decreased levels of Aβ 40 and 42 peptides and amyloid plaque load. Decreased Aβ peptide levels in aging APPSwe-PS1ΔE9 mice in the EP2−/− background could occur as a result of decreased Aβ peptide generation, decreased amyloid deposition, or increased clearance of Aβ peptides. Aged APPSwe-PS1ΔE9 mice lacking the EP2 receptor show significant decreases in levels of β-CTF, the product of BACE1 cleavage of APP. Decreased production of β-CTF peptide could result from decreased BACE1 or increased γ-secretase activities, or increased α-secretase activity. An increase in

γ-secretase activity is not likely in this model because mutant presenilin 1, particularly the PS1ΔE9 mutant, has maximal γ-secretase activity (Borchelt et al., 1997; Jankowsky et al., 2004). Moreover, an increase in γ-secretase activity in the EP2−/− background would result in lower levels of β-CTF but higher levels of Aβ peptides, and deletion of the EP2 receptor leads to significant decreases in both β-CTF and Aβ peptides (Figs. 2, 4). An increase in α-secretase activity is also unlikely because quantification of α-CTFs in APPSwe-PS1ΔE9 mice lacking the EP2 receptor shows no significant differences between EP2 backgrounds. Thus, in the APPSwe-PS1ΔE9 model, our findings suggest an effect of the EP2 receptor on BACE1 production of β-CTF, in which a functional EP2 receptor favors increased generation of β-CTF and Aβ peptides (supplemental Fig. 5, available at www.jneurosci.org as supplemental material).

The EP2 receptor does not alter β-CTF and Aβ peptide production in EP2 wild-type and null mice or young preplaque APPSwe-PS1ΔE9 mice in the absence of oxidative stress. The EP2 effect thus appears to be indirect, occurring from an interaction between the EP2 receptor and the APPSwe-PS1ΔE9 transgenic background, and is associated with increased oxidative stress that becomes significant in aged, but not young, APPSwe-PS1ΔE9 mice. Previous studies have shown that the EP2 receptor is a critical mediator of the microglial CD14-dependent inflammatory oxidative response (T. J. Montine et al., 2002). Because the inflammatory response to Aβ peptide partly involves the CD14 immune response (Fassbender et al., 2004; Milatovic et al., 2004), and Aβ peptide can induce microglial production of superoxide (Bianca et al., 1999), one hypothesis that emerges is that EP2-mediated oxidative stress indirectly promotes increased Aβ peptide levels via increased BACE1 processing. Recent investigations have linked increases in oxidative stress to increases in BACE1 activity and production of β-CTF (Tamagno et al., 2002; Apelt et al., 2004). Increases in BACE1 processing have also been demonstrated in aging and AD, in which inflammation and oxidative stress are prominent features (Fukumoto et al., 2002, 2004; Yang et al., 2003; Apelt et al., 2004; Holsinger et al., 2004). The increases in β-CTF seen in aged, but not young, APPSwe-PS1ΔE9 mice suggest an indirect effect of the EP2 receptor on BACE1 processing and generation of Aβ peptide through its promotion of the microglial oxidative response. The mechanisms by which increased oxidative stress leads to increased BACE1 activity are not well understood but could include posttranslational modifications of BACE1 or altered trafficking of BACE1 or its APP substrate (Cordy et al., 2003; Marlow et al., 2003; von Arnim et al., 2004; Sidera et al., 2005).

The present findings do not exclude the possibility of increased proteolytic degradation of the β-CTF fragment in the EP2−/−background. The mechanism(s) and magnitude of β-CTF breakdown independent of γ-secretase processing are not well established. Of relevance to this question is a study examining a transgenic model in which the β-CTF is overexpressed (Rutten et al., 2003); this model harbors elevated levels of β-CTF that are only modestly processed to Aβ peptide, suggesting that β-CTF may be relatively stable *in vivo*. In APPSwe-PS1ΔE9 mice, in which maximal γ-secretase activity occurs because of the PS1ΔE9 mutation, it is likely that proteolytic degradation of the β-CTF may represent only a minor component of its metabolism. In addition, this study does not address a secondary mechanism by which the EP2 receptor could promote elevated Aβ peptide levels, namely via enhanced deposition of Aβ peptides, a process that is accelerated in the setting of increased oxidative stress. Increases in oxidative stress have been suggested to precede and possibly trigger the deposition and accumulation of Aβ peptide into plaque (Pratico et al., 2001). Because we measured total guanidine-extracted Aβ peptides, representing all forms of Aβ peptides, including soluble, oligomeric, and fibrillar forms, the contribution of EP2-oxidant stress to increased fibrillization of Aβ peptides cannot be precisely determined.

Our findings suggest a potential mechanism for the antiamyloid and proamyloid properties of NSAIDs and COX-2 overexpression, respectively, in transgenic models of mutant APP (Lim et al., 2001; Jantzen et al., 2002; Xiang et al., 2002; Yan et al., 2003). The effects of EP2 receptor deletion may also be relevant to the preventive effects of chronic NSAID use against the development of AD (McGeer et al., 1996; Stewart et al., 1997; in t' Veld et al., 2001), in which decreased COX activity might lead to lower levels of downstream PGE_2 production and EP2 receptor signaling. However, it is important to note that several non-COX-related effects of NSAIDs have been demonstrated, including effects of NSAIDs on transcription factors important in the inflammatory response, such as NF-κB (nuclear factor κB) and AP-1 (for review, see Tegeder et al., 2001). In addition, recent *in*

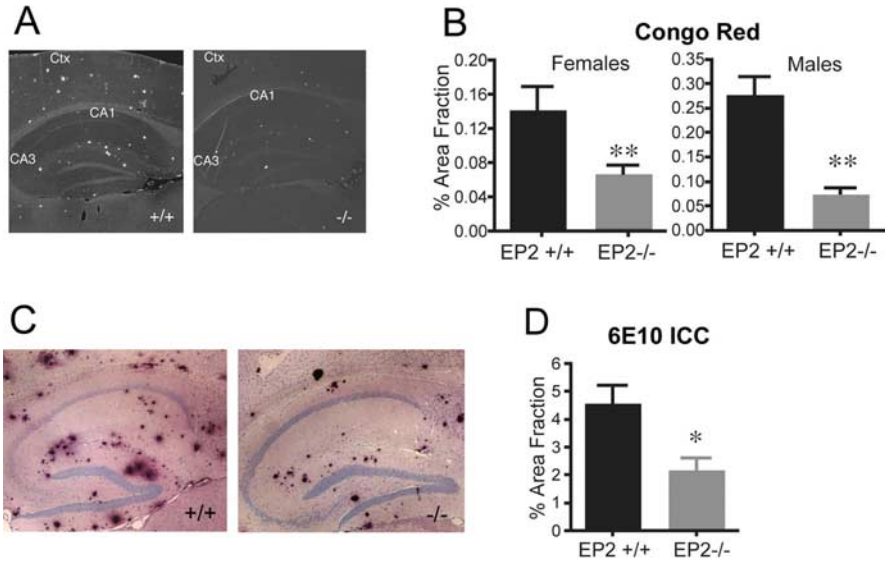

Figure 3. Deposited amyloid is reduced in APPSwe-PS1ΔE9 mice lacking the EP2 receptor. ***A***, Representative Congo red fluorescent images of rostral hippocampus in APPSwe-PS1ΔE9 mice in EP2+/+ and EP2−/− backgrounds (+/+ and −/−, respectively). Note the decrease in fluorescent Congo red signal in EP2−/− background in hippocampus (CA1 and CA3 subregions) and in overlying cerebral cortex (Ctx). ***B***, Quantification of Congo red fluorescence in 8-month-old female and 12-month-old male cohorts of APPSwe-PS1ΔE9 mice in EP2+/+ and EP2−/− backgrounds. Deletion of the EP2 receptor results in a marked decrease in the percentage area fraction occupied by Congo red fluorescence (**$p < 0.01$; $n = 7$–10 mice per gender per genotype, using 8–10 slides per brain). ***C***, Representative sections of rostral hippocampus from 8-month-old female APPSwe-PS1ΔE9 mice in EP2+/+ and EP2−/− backgrounds immunostained with anti-Aβ 6E10 antibody. ***D***, Quantification of stereologic point counting of 6E10 immunostained sections from cohorts of 8-month-old females demonstrate a marked decrease of amyloid load in the EP2−/− background. Every 10th section was immunostained for human Aβ peptide using monoclonal antibody 6E10 ($n = 8$–12 sections per brain). A significant decrease in total 6E10 immunostained area fraction was found in the EP2−/− background (*$p = 0.02$; $n = 6$–8 mice per genotype). Error bars represent SEM.

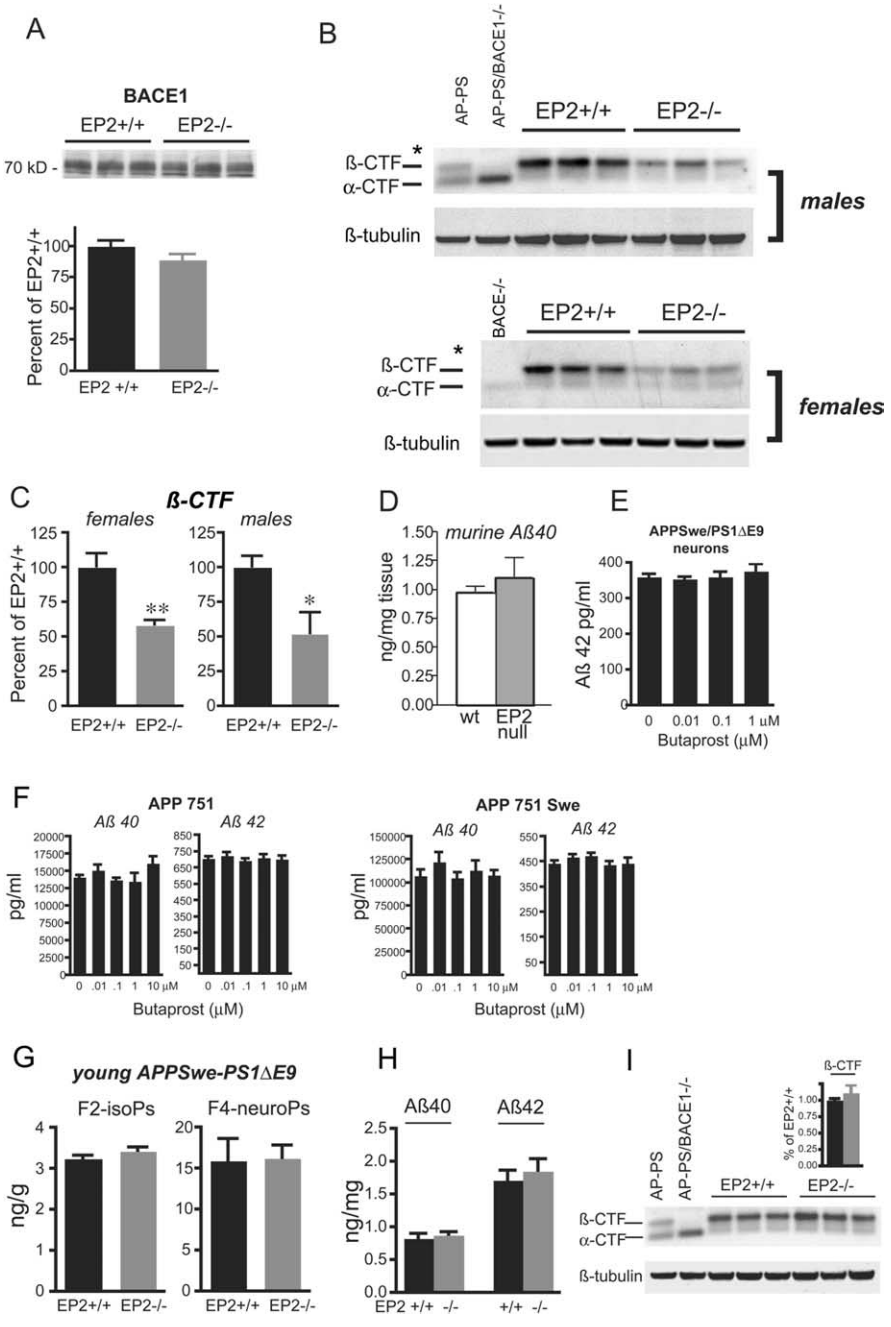

Figure 4. Deletion of the EP2 receptor leads to reduction in levels of β-CTF in aged, but not young, APPSwe-PS1ΔE9 mice. ***A***, Representative Western blot analysis (top) of BACE1 protein in male APPSwe-PS1ΔE9 mice in EP2$-/-$ and $+/+$ backgrounds does not show significant differences between EP2$+/+$ and $-/-$ backgrounds. Densitometry of BACE1 signal by quantitative Western blot analysis (bottom) does not show significant differences between EP2 backgrounds in males ($n = 4-5$ pairs). Similar results were found in female cohorts (data not shown). ***B***, Representative Western blot analyses of male and female APPSwe-PS1ΔE9 mice in EP2$-/-$ and $+/+$ backgrounds probed with anti-C-terminal APP antibody demonstrate a significant difference in levels of β-CTF between EP2 backgrounds. For male cohorts (top pair of blots; the top panel probed with anti-C-terminal APP antibody, the bottom panel probed with β-tubulin antibody as a loading control), control APPSwe-PS1ΔE9 and APPSwe-PS1ΔE9/BACE1$-/-$ lysates (derived from older 24-month-old mice, kindly provided by Dr. Phil Wong) were run as positive controls to confirm the identity of the β-CTF and α-CTF. There is no β-CTF band in the APPSwe-PS1ΔE9/BACE1$-/-$ lane, only the α-CTF. For female cohorts (bottom pair of blots), a control lane was run from BACE1 $-/-$ mice (lane 1), in which the β-CTF band is also absent. The asterisk denotes 14 kDa marker. ***C***, Densitometry of β-CTF autoradiographic signal demonstrates a significant reduction in levels of β-CTF in APPSwe-PS1ΔE9 mice lacking the EP2 receptor (*$p < 0.05$ in males; **$p < 0.01$ in females; $n = 4-5$ pairs). ***D***, Aβ 40 levels in EP2 null and wild-type mice do not differ between genotypes, indicating that the EP2 receptor does not directly modulate APP processing ($n = 4-6$ pairs of female mice, 4–6 months of age). ***E***, Pharmacologic activation of the EP2 receptor with the selective agonist butaprost in primary APPSwe-PS1E9 cortical neurons does not alter levels of Aβ 42. Neurons (8 d *in vitro*) were stimulated with butaprost (10 nM to 1 μM) for 72 h ($n = 6$ wells per condition). Aβ40 levels were below the limit of detection (<20 pg/ml). ***F***, Stimulation of EP2 receptor with butaprost in APP751 or in APP751Swe-expressing CHO cells does not alter Aβ 40 or 42 processing, suggesting that EP2 receptor does not directly alter APP processing *vitro* studies demonstrate a novel non-COX-dependent activity of selected NSAIDs, which, at high concentrations, can directly alter γ-secretase activity and reduce the ratio of Aβ 42/38 peptides in cultured cells (Weggen et al., 2001). These interesting findings have identified lead compounds acting directly on γ-secretase to favor shorter forms of Aβ peptide; however, studies have differed on whether this mechanism occurs *in vivo* (Eriksen et al., 2003; Lanz et al., 2005), particularly at the lower therapeutic doses that result in reduced risk of developing AD and the low penetration of NSAIDs across the blood–brain barrier (Mannila et al., 2005).

An important line of defense in the CNS against infection, injury, or dysregulated Aβ metabolism is the innate immune response, in which microglia become activated and able to phagocytose the offending agents. Recently, we observed that primary cultures of microglia from EP2$-/-$ mouse cerebrum acutely displayed enhanced phagocytosis of synthetic Aβ 42 and increased *ex vivo* clearance of Aβ 40 and 42 from hippocampal slices of patients who died of AD (Shie et al., 2005). Although the present study does not examine microglial phagocytosis in aging cohorts of APPSwe-PS1ΔE9 mice, a potential additional role for the EP2 receptor in microglial clearance of Aβ peptides should be investigated. Recent *in vitro* studies demonstrate that the EP2 receptor inhibits phagocytosis of bacterial components by lung alveolar macrophages (Aronoff et al., 2004), and PGE_2 inhibits phagocytosis by macrophages by a process that is dependent on increased cAMP levels (Hutchison and Myers, 1987; Canning et al., 1991; Borda et al., 1998; Aronoff et al., 2004). Conversely, NSAIDs have been shown to potentiate phagocytosis by macrophages (Bjornson et al., 1988; Laegreid et al., 1989; Gilmour et al., 1993; Gurer et al., 2002), a function that is COX

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($n = 6$ wells per condition). ***G***, Young 2-month-old female APPSwe-PS1ΔE9 mice in EP2$-/-$ and $+/+$ backgrounds do not demonstrate differences in levels of F2-isoprostanes (isoPs) or F4-neuroprostanes (neuroPs) ($n = 4-6$ pairs of female mice). ***H***, The same 2-month-old female APPSwe-PS1ΔE9 mice in EP2$-/-$ and $+/+$ backgrounds do not demonstrate differences in levels of Aβ 40 or Aβ 42 ($n = 4-6$ pairs of female mice). ***I***, Quantitative Western blot analysis of β-CTF in the same 2-month-old mice does not show differences in levels of β-CTF between EP2 backgrounds. A representative Western blot probed with antibody to the C terminus to APP (as is ***B*** above) is shown. Graph shows no difference in β-CTF densitometry ($n = 4$ pairs of female mice). Error bars represent SEM.

dependent (Canning et al., 1991). Together, these findings and those of the present study suggest a dual role for the EP2 receptor in the APPSwe-PS1ΔE9 model of amyloidosis. EP2 signaling in this model not only may promote an inflammatory oxidative response with an associated increase in levels of Aβ peptide from increased BACE1 processing but may also inhibit phagocytosis and clearance of accumulating Aβ peptides. The precise relationship between microglial activation with production of cytotoxic compounds and phagocytosis is not completely understood, but the potential role of the EP2 receptor in both microglial activation and phagocytosis suggests that the EP2 receptor functions upstream, possibly as a regulator of both processes.

In AD and murine models of familial AD, a pathological hallmark is the development of inflammation in the setting of accumulation of Aβ peptides. An important question in the *in vivo* development of pathology is whether oxidant production as well as defective phagocytosis from EP2 receptor signaling lead to additional inflammation, oxidative damage, and secondary neurotoxicity. An additional question is whether EP2-mediated oxidative damage contributes to even earlier stages of pathogenesis, namely the shift from monomeric to pathologic oligomeric forms of Aβ peptides (Walsh and Selkoe, 2004). Our findings on the role of the EP2 receptor in this model of familial AD provide a rationale for additional investigation of anti-inflammatory therapeutics targeting the EP2 receptor. The recent discovery of cardiovascular complications from long-term use of specific COX-2 inhibitors underscores the complexity of the prostaglandin response. Clearly, identification of downstream prostaglandin pathways that function in specific disease paradigms should assist in developing more selective anti-inflammatory therapies preferable to the broad actions of COX-2 inhibitors and NSAIDs.

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