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PGH₂-derived levuglandin adducts increase the neurotoxicity of amyloid β _{1–42}

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Abstract

The body of evidence indicating that oligomers of amyloid β _{1–42} ($A\beta$ _{1–42}) produce toxicity to neurons, together with our demonstration that prostaglandin H₂ (PGH₂) oligomerizes amyloid β _{1–42}, led to the examination of the neurotoxicity of amyloid β _{1–42} treated with PGH₂. The neurotoxic effects of $A\beta$ _{1–42} incubated with PGH₂ was examined in primary cultures of cerebral neurons of mice, monitoring the reduction of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as an indicator of cell toxicity. Whereas $A\beta$ _{1–42} itself, incubated for 24 h, has little or no effect on MTT reduction, $A\beta$ _{1–42} 24 h after exposure to PGH₂ produced a marked inhibition of MTT reduction, comparable with the inhibition resulting from $A\beta$ _{1–42} that has been oligomerized by incubation for 6 days. Similar results were obtained when $A\beta$ _{1–42} was incubated with levuglandin E₂ (LGE₂), a reactive aldehyde formed by spontaneous rearrangement of PGH₂. The oligomers formed from reaction of $A\beta$ _{1–42} with LGE₂ exhibit immunochemical similarity with amyloid-derived diffusible ligands (ADDLs), as determined by analysis of the products of reaction of $A\beta$ _{1–42} with LGE₂ using western blotting with an antibody that is selective for ADDLs.

Keywords

amyloid; amyloid-derived diffusible ligands; cyclooxygenase; levuglandin; neurotoxicity; prostaglandin H

A contribution of cyclooxygenase (COX) activity to the pathogenesis of Alzheimer's disease (AD) may be inferred from two large prospective studies on individuals with no baseline dementia that found a 60–80% reduction in risk of developing AD associated with the use of non-steroidal anti-inflammatory drugs for at least 2 years (Stewart et al. 1997; in 't Veld et al. 2001). An alternative explanation of these results is derived from the finding that some non-steroidal anti-inflammatory drugs (NSAIDs) used at high concentrations have been shown to exert COX-independent effects, including alteration of γ -secretase cleavage of the amyloid-precursor protein (Weggen et al. 2001, 2003; Eriksen et al. 2003). The finding of a fivefold elevation of prostaglandin E₂ (PGE₂) in cerebrospinal fluid of patients with AD reflects increased COX activity in AD (Montine et al. 1999). A link between COX activity and loss of cognitive function is also consistent with data from animal models of dementia. Transgenic mice overexpressing human COX-2 (hCOX-2) in brain develop an age-related loss of cognitive function that is reversed by celecoxib (Andreasson et al. 2001). In a transgenic mouse model

of AD with mutations of the amyloid precursor protein (APPswe) and presenilin-1 (A246E), amyloid plaque formation is increased by overexpression of hCOX-2 (Xiang et al. 2002). Moreover, treatment with the non-specific COX inhibitor, ibuprofen, suppresses amyloid plaque formation and ameliorates behavioral changes in an APPswe (Tg2576) transgenic mouse (Lim et al. 2000, 2001).

The product of both COX isoforms is the endoperoxide, prostaglandin H₂ (PGH₂), which may then serve as a substrate for enzymes that yield prostaglandins and thromboxane A₂ as products. PGH₂ also rapidly undergoes rearrangement to PGE₂ and PGD₂, and via ring cleavage to secoprostaglandins that are γ -keto-aldehydes (Salomon et al. 1984). These γ -keto-aldehydes, designated levuglandin (LG) E₂ and LGD₂, constitute about 20% of the rearrangement products of PGH₂ (Salomon et al. 1984; Boutaud et al. 1999). The γ -keto-aldehydes are also formed by free radical oxidation of arachidonic acid *in vitro* (Brame et al. 1999) and in cell membranes following oxidant injury (Brame et al. 2004). The γ -keto-aldehyde structure is highly reactive and readily forms adducts with amine groups. By adducting the ϵ -amine of lysine or the N-terminal α -amine, LGs form covalent adducts on proteins (Iyer et al. 1994). Importantly, the initial lysyl-levuglandin adducts themselves are also highly reactive; the Schiff base adduct with lysine can undergo nucleophilic attack (Harada 1970) and the oxidation of the pyrrole adducts generates sites of electrophilic reactivity (Amarnath et al. 1994, 1998). Thus, lysyl-LG adducts can form covalent bonds with other nucleophiles, leading to intermolecular crosslinking. Such crosslinking has been demonstrated between proteins (Iyer et al. 1989; Boutaud et al. 2001).

The spontaneous and reversible association of amyloid β (A β) into dimers led us to consider that this dimerization would energetically favor the crosslinking of A β by levuglandins. Accordingly, we examined the effect of PGH₂ on A β ₁₋₄₂, and demonstrated that PGH₂ greatly accelerated the formation of dimers and higher oligomers (Boutaud et al. 2002b). The same marked increase in the rate of formation of oligomers of A β was produced by synthetic LGE₂. The consequences of accelerated formation of stable A β oligomers upon reaction with PGH₂ have now been examined by assessing whether the resulting oligomers of A β ₁₋₄₂ are neurotoxic. For this purpose, the effect of the oligomers of A β produced by PGH₂ and by LGE₂ on the viability of neurons in primary culture was examined.

Material and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum and N2 neuron supplement were purchased from Gibco (Rockville, MD, USA). A β ₁₋₄₂ was purchased from Sigma (St Louis, MO, USA). PGH₂ and LGE₂ were prepared as described previously in Boutaud et al. (2002a) and Amarnath et al. (2005), respectively.

Primary cell culture of neurons

Primary dissociated cultures of neurons were prepared according to methods described in *Culturing Nerve Cells* by Gary Banker and Kimberly Goslin (Banker and Goslin 1998). Wild-type mice on the C57Bl/6J background were used for the neurotoxicity experiments. The pregnant mouse (embryonic day 15, E15) was killed, then the uterus was removed and placed in a sterile Petri dish. The remaining procedures were performed in a laminar flow hood. Brains from E15 mice were dissected under aseptic conditions while immersed in calcium- and magnesium-free Hank's Balanced Salt Solution (HBSS); the cerebral cortex with meninges was removed, transferred to another dish and again immersed in HBSS. All the cerebral cortices from one litter were transferred to a 50 mL conical tube to which 9 mL HBSS and 1 mL 2.5% trypsin were added. After incubation for 15 min at 37 °C, the trypsin-containing solution was

removed and replaced by 10 mL HBSS for 5 min. This step was repeated two times. Tissues were then dissociated by triturating first with a Pasteur pipette and then with a Pasteur pipette with the tip fire-polished to about one half the normal diameter. Trituration was continued until no chunks of tissue remained. Cell density was determined with a hemacytometer, and the number of viable cells assessed by Trypan blue exclusion. Cells were then distributed onto poly-D-lysine-coated 12-well plates at a density of 3×10^5 cells/cm² and maintained in DMEM supplemented with 10% fetal calf serum and N2 (Gibco) neuron supplement. Growth medium was changed once weekly and cultures were used between 14 and 16 days *in vitro*.

Effect of PGH₂ on the neurotoxicity of A β

A β ₁₋₄₂ 35 μ M, in phosphate-buffered saline (PBS) containing 200 μ M EDTA, was incubated for 24 h with PGH₂ at a concentration that provides LGE₂/D₂ in a molar equivalent to each lysine of A β ₁₋₄₂, or with the same volume of vehicle (acetone). A β ₁₋₄₂ was also incubated for 6 days at 22°C under the same conditions as above to generate the neurotoxic species describes as 'aged' amyloid (Simmons et al. 1994; Landolfi et al. 1998). After incubation, 100 μ L of the different solutions of A β ₁₋₄₂ were pre-incubated at 22°C with 10 μ L 10 $\times$ growth medium for 2 min in order to 'pre-adduct' any undegraded LGE₂, and added to mouse primary cerebral neurons for determination of the neurotoxicity.

Effect of LGE₂ on the neurotoxicity of A β

Because of the potential neurotoxicity of PGH₂ itself, similar experiments were performed by incubating A β ₁₋₄₂ with LGE₂. A β ₁₋₄₂ 35 μ M in PBS containing 200 μ M EDTA was incubated for 24 h with 0.2 molar equivalents of synthetic LGE₂, or with the same volume of vehicle (ethanol). A β ₁₋₄₂ in PBS containing 200 μ M EDTA was also incubated for 6 days at 22°C to allow formation of toxic oligomers. Because LGE₂ might harbor cellular toxicity by itself, we included a control condition in which synthetic LGE₂ was incubated in PBS containing 200 μ M EDTA for 24 h. After incubation, an aliquot was saved for analysis of oligomers by western blot, and 100 μ L of the different solutions of A β ₁₋₄₂ were pre-incubated at 22°C with 10 μ L 10 $\times$ growth medium for 2 min in order to 'pre-adduct' any undegraded LGE₂; these were then added to mouse primary cerebral neurons for determination of the neurotoxicity.

Neurotoxicity experiments

At day 14 of culture, 100 μ L of the different solutions of A β ₁₋₄₂ in PBS containing 200 μ M EDTA were mixed with 10 μ L 10 $\times$ medium and added to the cells. After 24 h, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) 2.5 mg/mL in water was added to the cells for a final concentration of 250 μ g/mL and incubated at 37°C for 2 h. At this time, the medium was removed and the purple solid solubilized in 100 μ L HCl 0.1 N in isopropanol. The absorbance at 570 nm was monitored in a spectrophotometer.

Analysis of A β ₁₋₄₂ oligomers by SDS-PAGE

The presence of oligomers in the solutions of A β ₁₋₄₂ incubated with ethanol or 0.1 molar equivalents of synthetic LGE₂ for 24 h, or incubated for 6 days, was analyzed by western blot. After incubation, 5 μ g peptide were diluted with sodium dodecyl sulfate (SDS) loading buffer containing 15% β -mercaptoethanol and denatured at 70°C for 10 min. After separation by SDS-polyacrylamide gel electrophoresis (PAGE) using 4–12% Tris-Tricine gels, the proteins were transferred onto nitrocellulose membranes 0.2 μ m (Bio-Rad Laboratories, Hercules, CA, USA) and immediately boiled for 30 min in Tris-buffered saline. The proteins were then analyzed by western blot with a polyclonal A β ₁₋₄₂ antibody (Zymed Laboratories, South San Francisco, CA, USA). Proteins were visualized by horseradish peroxidase-catalyzed chemiluminescence (NEN Life Sciences, PerkinElmer, Boston, MA, USA). In parallel experiments, A β ₁₋₄₂ was incubated with concentrations of PGH₂ that provide levuglandins in

0.05 or 0.1 molar equivalent to each amyloid. After 24 h at 22°C, 0.8 µg Aβ₁₋₄₂ was diluted with SDS loading buffer and the proteins were analyzed by western blot as described above.

Analysis of amyloid-derived diffusible ligands (ADDLs) by western blot

Aβ₁₋₄₂ at 8.8 µM was incubated with ethanol or LGE₂ in 1 or 0.1 molar equivalent at 22°C in sodium phosphate buffer, pH 7.4, with 200 µM EDTA for 24 h. After incubation, 0.25 µg peptide was diluted with SDS loading buffer. No heat was applied to the samples prior to loading on the gel. The proteins were separated by SDS-PAGE using 16.5% Tris-tricine gels (Bio-Rad), and were transferred onto nitrocellulose membranes 0.2 µm (Bio-Rad) at 100 V for 60 min at 4°C. The proteins were then analyzed by western blot with a polyclonal antibody specific for ADDLs (M90.2) as described previously (Lambert et al. 2001). Proteins were visualized by horseradish peroxidase-catalyzed chemiluminescence (NEN Life Sciences).

Preparation of ADDLs

Aβ₁₋₄₂ peptide (California Peptide Research, Napa, CA, USA) was used to prepare synthetic ADDLs according to published protocols (Chang et al. 2003).

Results

The aim of this investigation was to test the hypothesis that Aβ₁₋₄₂ oligomers formed by reaction with PGH₂ were neurotoxic. The neurotoxic effects of PGH₂-treated Aβ₁₋₄₂ on mouse cerebral neurons in primary cultures was determined by monitoring the reduction of MTT (Shearman et al. 1994, 1995; Hughes et al. 2000; Woltjer et al. 2003). Aβ₁₋₄₂ was incubated for 24 h with or without PGH₂ as described in the previous studies in which oligomers were formed within this time period upon exposure to PGH₂ (Boutaud et al. 2002b). Whereas Aβ₁₋₄₂ itself incubated for 24 h had no effect on MTT reduction (Fig. 1), Aβ₁₋₄₂ 24 h after exposure to PGH₂ produced a marked inhibition of MTT reduction that was comparable to the neurotoxicity known to result from aggregated amyloid formed by incubation of Aβ₁₋₄₂ for 6 days (Simmons et al. 1994; Landolfi et al. 1998).

Because PGH₂ can rearrange to form PGE₂ and PGD₂ in addition to LGE₂, the same experiments were carried out using synthetic LGE₂. Also, LGE₂ alone was used as a control to exclude direct neurotoxicity, which is considered unlikely after 24 h in the incubation buffer followed by exposure to serum containing amino acids that should 'pre-adduct' any undegraded LGE₂ before it is added to the neurons. As seen in Fig. 2(a), incubation of Aβ₁₋₄₂ with LGE₂ for 24 h produced the same inhibition of MTT reduction as Aβ₁₋₄₂ incubated for 6 days. By comparison, Aβ₁₋₄₂ incubated alone for 24 h had no more effect than PBS or LGE₂ added alone to the cells.

The species of Aβ present in the conditions associated with neurotoxicity were assessed by western blot analysis after heat denaturation of the peptides in the presence of SDS. In the absence of LGE₂, no oligomers were detected by western blot after 24 h, whereas oligomerization was extensive after LGE₂ was added for 24 h (Fig. 2b). Similar oligomerization was observed when Aβ₁₋₄₂ was incubated in PBS for 6 days (Fig. 2b). This electrophoretic mobility of the oligomers is the same as the mobility observed when Aβ₁₋₄₂ was incubated with PGH₂ under the same conditions (Boutaud et al. 2002b). Using western blotting with an antibody that is selective for ADDLs (Chang et al. 2003; Gong et al. 2003), we determined that the products of reaction of LGE₂ with Aβ₁₋₄₂ exhibit immunochemical similarity with ADDLs, and that formation of these oligomers occurred at a molar ratio as little as 0.1 LGE₂ per Aβ₁₋₄₂ (Fig. 3). In these experiments, the samples were not denatured by heat prior to gel migration. It is important to note at this point that when analyzed in mild denaturing conditions (no heat applied to the samples), traces of oligomers were detected in the control conditions

by the ADDL-specific antibody (Fig. 3), whereas no oligomers were detected under the same conditions when analyzed by western blot following strong denaturing conditions (Fig. 2b). In contrast, incubation of A β ₁₋₄₂ with LGE₂ in a molar ratio LGE₂/A β ₁₋₄₂ as low as 0.05 yielded oligomers that were heat resistant. Similar observations were made when analyzed by electron microscopy, where large fibrils were observed under non-denaturing conditions after 24 h whereas no aggregates were observed by western analysis (Boutaud et al. 2002b). This suggests that oligomers formed spontaneously in 24 h are sensitive to temperature, but that reaction of A β ₁₋₄₂ with LGE₂ induces a change in the structure of the oligomers formed in 24 h, rendering them resistant to denaturation by heat.

Discussion

Soluble oligomers of A β are increased in the brain in Alzheimer's disease (Kuo et al. 1996; Hardy and Selkoe 2002; Gong et al. 2003) and these oligomers are neurotoxic (Roher et al. 1996; Lambert et al. 1998; Hartley et al. 1999; Dahlgren et al. 2002; Walsh et al. 2002; Stine et al. 2003; Lacor et al. 2004). We have previously demonstrated that the addition of PGH₂ or LGE₂ to A β markedly accelerates formation of soluble oligomers that have the ultrastructural features of A β -derived diffusible ligands (Boutaud et al. 2002b). We now provide evidence that these oligomers are neurotoxic and are recognized by ADDL selective antibodies. These results provide a new molecular link between COX activity and the accumulation of soluble oligomers of A β in AD brain.

Although A β ₁₋₄₂ oligomers have been implicated in memory loss, the understanding of mechanisms that contribute to oligomer formation is still evolving. One proposed mechanism is the covalent modification of the A β ₁₋₄₂ peptide following oxidative stress catalyzed by metals such as copper (Atwood et al. 2000; Bush et al. 2003; Sparks and Schreurs 2003). We present evidence for a new mechanism of A β ₁₋₄₂ oligomerization which proceeds through formation of covalent adducts of reactive lipids generated by rearrangement of the product of cyclooxygenase.

To ascertain that oligomerization of A β by LGE₂ occurred by a mechanism that was independent of divalent metal cations, we performed the experiments in a buffer containing 200 μ M EDTA; in these conditions we were not able to observe toxicity or SDS-stable oligomers after 24 h of incubation. PGH₂ rearranges not only to LG but also to PGE₂ and PGD₂; PGE₂ and PGD₂ can be dehydrated to PGA₂ and PGJ₂, respectively, and the PGE₂ and PGA₂ series is known to cause neurodegeneration in neuroblastoma cells (Prasad et al. 1998). The potentially confounding effects of these non-LG rearrangement/dehydration products of PGH₂ were addressed by demonstrating that A β ₁₋₄₂ incubated with LGE₂ itself is neurotoxic. Whereas the PGH₂ used in these studies has the stereochemical characteristics of that formed enzymatically by the COXs, the synthetic LGE₂ comprised several isomers. This indicates that the same neurotoxicity would be expected from iso-levuglandins derived from the series of PGH₂ isomers as results from free radical-mediated lipid peroxidation.

Oligomerization of A β by LGE₂ occurs with ratios of LGE₂ : A β of only 1 : 10. This suggests that intermolecular crosslinking cannot be the sole mechanism for oligomerization, and raises the possibility that lipid modification of A β serves as a seed to accelerate oligomerization, analogous to the lipid modification of A β by GM1 ganglioside (Hayashi et al. 2004) or by cholesterol-derived molecules (Zhang et al. 2004).

This demonstration that LGE₂ induces formation of neurotoxic oligomers of A β ₁₋₄₂ *in vitro* occurs in the context of evidence that COX activity can engender formation of LG adducts of protein in cells and *in vivo*. This includes our finding that activation of platelets produces LG adducts of platelet proteins that is COX-1-dependant (Boutaud et al. 2003), and that overexpression of hCOX-2 in mouse neurons increases the formation of LG adducts of protein

in brain (Boutaud et al. 2005). More recently, we have demonstrated that levels of LG adducts of protein in the hippocampus of patients with AD are increased 12-fold in comparison with age-matched controls (Zagol-Ikapitte et al. 2005). Moreover, the levels of LG adducts of protein are highly correlated with the pathological evidence of AD severity. These findings in concert provide a rationale for investigations that examine the consequences of COX-dependent oligomerization of A β in AD, and in animal models that simulate AD.

Previously, the cellular and biochemical significance of prostanoids has been framed in terms of the stable agents, such as PGE₂, PGF_{2 α} and PGD₂, and their reversible interactions with G-protein receptors. The evidence presented in this report lays the foundation for a new biochemical paradigm for the participation of the cyclooxygenase enzymes in Alzheimer's disease.

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Abbreviations used

A β , amyloid β
 AD, Alzheimer's disease
 ADDLs, amyloid-derived diffusible ligands
 COX, cyclooxygenase
 DMEM, Dulbecco's modified Eagle's medium
 E, embryonic day
 HBSS, Hank's balanced salt solution
 LG, levuglandin
 MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide
 NSAID, non-steroidal anti-inflammatory drug
 PBS, phosphate-buffered saline
 PG, prostaglandin

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Fig. 1.

PGH₂ accelerates the neurotoxic properties of A β ₁₋₄₂. A β ₁₋₄₂ in PBS was incubated for 24 h with PGH₂ at a concentration that provides LGE₂/D₂ in a molar equivalent to each lysine of A β ₁₋₄₂, or with the same volume of vehicle (acetone). A β ₁₋₄₂ in PBS was also incubated for 6 days at 22°C to allow formation of oligomers. After incubation, 100 μ L of the different solutions of A β ₁₋₄₂ or of PBS were mixed with 10 μ L 10 $\times$ growth medium and added to mouse primary cerebral neurons at day 14 of culture in 96-well plates. After 24 h, MTT 2.5 mg/mL in water was added to the cells for a final concentration of 250 μ g/mL and incubated at 37°C for 2 h. At this time, the medium was removed and the purple crystals were solubilized in 100 μ L HCl 0.1 N in isopropanol. The absorbance at 595 nm was monitored in a spectrophotometer. Each data point represents the mean $\pm$ SEM of six values; * p < 0.01; ** p < 0.005.

Fig. 2.

LGE₂-derived oligomers of A β_{1-42} are neurotoxic. A β_{1-42} in PBS was incubated for 24 h with 0.2 molar equivalents of synthetic LGE₂ or with the same volume of vehicle (ethanol).

A β_{1-42} in PBS was also incubated for 6 days at 22°C to allow formation of oligomers, and synthetic LGE₂ was incubated in PBS for 24 h. (a) After incubation, 100 μ L of the solutions of A β_{1-42} , LGE₂ or PBS were mixed with 10 μ L 10 $\times$ growth medium and added to mouse primary cerebral neurons at day 14 of culture. After 24 h, MTT was added to the cells for a final concentration of 250 μ g/mL and incubated at 37°C for 2 h. At this time, the medium was removed and the purple crystals were solubilized in 100 μ L HCl 0.1 N in isopropanol. The absorbance at 595 nm was monitored in a spectrophotometer. Each data point represents the mean $\pm$ SEM of six values; ** p < 0.005. (b) After incubation, 5 μ g A β_{1-42} incubated as indicated in the figure were separated by SDS-PAGE using 4–12% Nupage gel, and the different species of A β_{1-42} were analyzed by western blot.

Fig. 3.

LGE₂ induces formation of oligomers of Aβ₁₋₄₂ that exhibit immunochemical similarity with ADDLs in a dose-dependant manner. Aβ₁₋₄₂ in PBS was incubated for 24 h with 1.0 or 0.1 molar equivalents of synthetic LGE₂, or with the same volume of vehicle (ethanol). After incubation, 0.25 μg Aβ₁₋₄₂ incubated as indicated in the figure was separated by SDS-PAGE using 16.5% Tris-tricine gel, and the different species of Aβ₁₋₄₂ were analyzed by western blot using an antibody specific for ADDLs (M90.2). A control solution of ADDLs was obtained as described previously (Lambert et al. 2001).