Cyclooxygenase-Dependent Lipid-Modification of Brain Proteins

Olivier Boutaud[1]; Katrin I. Andreasson[3]; Irène Zagol-Ikapitte[1]; John A. Oates[1, 2]

[1] Departments of Pharmacology, and [2]Medicine, Vanderbilt University, Nashville, Tenn.
[3] Departments of Neuroscience and Neurology, John Hopkins School of Medicine, Baltimore, Md.

Corresponding author:
Olivier Boutaud, PhD, Department of Pharmacology, Vanderbilt University, Nashville, TN 37232-6602, USA (E-mail: olivier.boutaud@vanderbilt.edu)

Substantial evidence indicates that both β-amyloid and cyclooxygenase activity contribute to the pathogenesis of Alzheimer disease. The immediate product of the cyclooxygenases, prostaglandin H_2, rapidly rearranges in aqueous solution, with approximately 20% being converted to levuglandins E_2 and D_2. These γ-ketoaldehydes are highly reactive and rapidly adduct to accessible amine groups on macromolecules, particularly the ε-amine of lysine residues on proteins. The immediate LG-lysine adducts are themselves reactive, and can covalently crosslink proteins. PGH_2, acting via LGs, accelerates the formation of the type of oligomers of amyloid β that has been associated with neurotoxicity. In this review, we discuss the cyclooxygenase-dependent lipid-modification of proteins by levuglandins in vitro, in cells in culture and in vivo in transgenic mice over-expressing COX in the brain.

Brain Pathology 2005;15:139-142.

INTRODUCTION

A link between cyclooxygenase (COX) and Alzheimer disease (AD) has been suggested by several lines of evidence. A contribution of COX activity to the pathogenesis of AD may be inferred from 2 large prospective studies of individuals with no baseline dementia that found 60% to 80% reduction in the risk of developing AD associated with the use of non-steroidal anti-inflammatory drugs for at least 2 years (18, 30). Moreover, a 5-fold elevation of PGE_2 in the cerebral spinal fluid of patients with AD reflects increased COX activity in the disease (26). A relationship between COX activity and loss of cognitive function also is consistent with data from animal models of dementia. Transgenic mice over-expressing human COX-2 (hCOX2) in brain developed an age-related loss of cognitive function (3) that is reversed by celecoxib (Melnikova and Andreasson, unpublished results). In a transgenic model of AD with mutations of the amyloid precursor protein (APP_{Swe}) and presenilin-1 (A246E), amyloid plaque formation is increased by over-expression of hCOX-2 (34). Moreover, treatment with the non-specific COX inhibitor, ibuprofen, suppresses amyloid plaque formation in an APP_{Swe} (Tg2576) mutant mouse (24). This concerted evidence prompts consideration of the consequences of COX activity in brain.

LEVUGLANDINYL-LYSINE LACTAM ADDUCTS ARE FORMED FOLLOWING COX ACTIVITY

The product of both COX isoforms is the endoperoxide PGH_2, which then may serve as a substrate for synthases and a reductase that yield prostaglandins and thromboxane A_2 as products (Figure 1). PGH_2 also rapidly undergoes non-enzymatic rearrangement to PGE_2 and PGD_2, and via ring cleavage, to secoprostaglandins that are γ-ketoaldehydes. These γ-ketoaldehydes have been designated levuglandin (LG) E_2 and LGD_2 and constitute ~20% of the rearrangement products of PGH_2 (5, 29). The γ-ketoaldehyde structure confers a high degree of reactivity on the LGs, which readily form adducts with amine groups.

Salomon et al (20) demonstrated that a pyrrole adduct is formed from the reaction of levuglandin E_2 with protein-based lysyl ϵ-amino groups, and Brame et al showed that oxidation of the pyrrole intermediate leads to formation of the more stable lactam and hydroxylactam structures (8). We

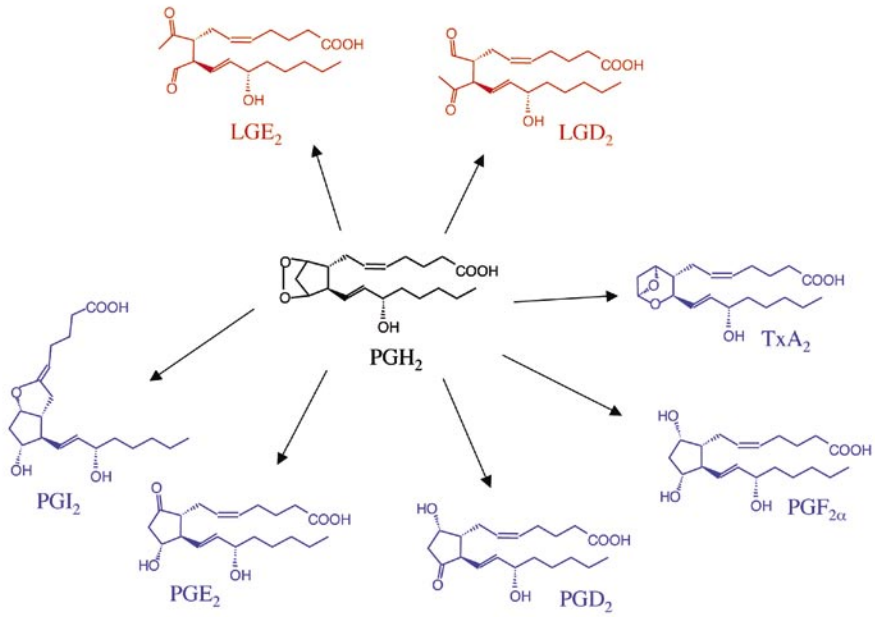

Figure 1. *Prostaglandins and levuglandins are formed from prostaglandin H_2.* PGH_2 is a substrate for several enzymes that metabolize it further into prostaglandins and thromboxane A_2 (blue pathway). PGH_2 rearrangement in aqueous solution will yield levuglandins (red pathway) as well as PGE_2 and PGD_2.

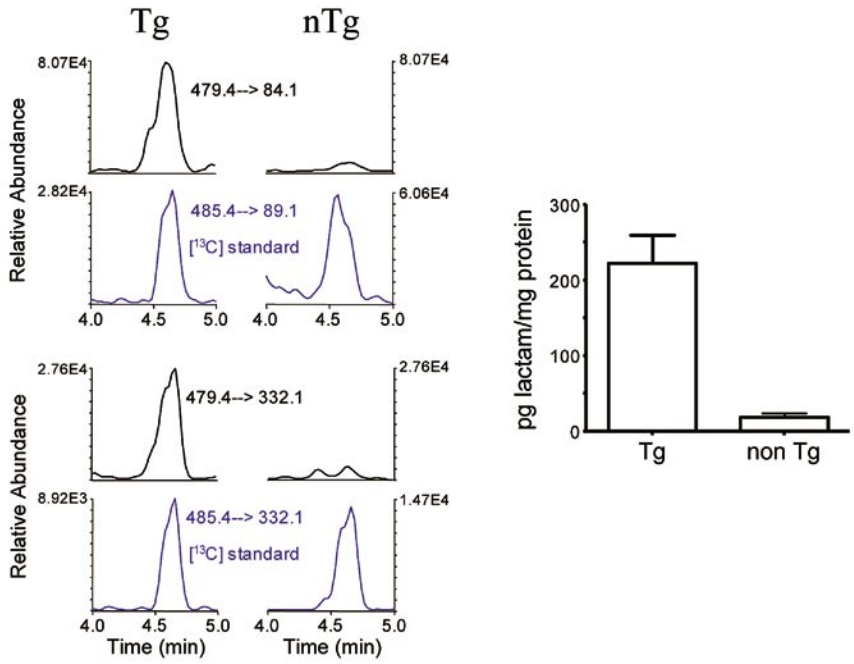

Figure 2. *hCOX-2 overexpression in mouse brain increases the formation of LG-lysine adducts in brain.* Transgenic mice (Tg) overexpressing hCOX-2 with a Thy-1 promoter (line 303) have increased levels of COX-2 in brain neurons (3). Proteins from brains of these transgenic and wild-type (nTg) mice were digested to single amino acids by step-digestion with pronase and aminopeptidase M. The LG-lysine lactam adducts were purified by chromatography on Oasis™ cartridge followed by a reverse phase HPLC, then analyzed by LC/ESI/MS/MS in the positive ion mode. Selected reaction monitoring of precursor ion at *m/z* 479 (LG-lysine lactam) produces daughter ions at *m/z* 84.1 and 332.1. An internal standard, [$^{13}C_6$] LG-lysine lactam, was added to the brain sample prior to the isolation procedure. Selected reaction monitoring of precursor ion at *m/z* 485 ([$^{13}C_6$] LG-lysine lactam standard) produces daughter ions at *m/z* 89.1 and 332.1. The peak for the daughter ion at *m/z* 332.1 was integrated in each sample. The amount of LG-lysine lactam present in tissues was determined using the area of the internal standard and corrected to the amount of protein. The values represent the mean ± S.E.M. The statistical analysis was performed using two way ANOVA ($p<0.01$; n = 9 for transgenic and n = 5 for non transgenic animals).

characterized a Schiff base that is formed more rapidly than the lactam when both LGE_2 and PGH_2 are reacted with free lysine in solution (5).

Levuglandins also can react with the guanidio group of arginine to form a *bis*-adduct that undergoes hydrolytic cleavage to yield *bis*-(levuglandinyl)urea (BLU) and ornithine (35). Although formation of BLU adducts using a synthetic peptide required a concentration of LGE_2 in excess of the concentration of lysine, BLU adducts are formed by reaction of LGE_2 with human serum albumin with a molar ratio of LGE_2 to free amines of 0.04. These results suggest that protein microenvironment could modify the reactivity of certain arginines, leading to the formation of BLU adducts in conditions where levuglandins are generated in small amounts. The reaction of LG with arginine residues in proteins provides the basis for a hypothesis that substitution of an ornithine for arginine as the result of cyclooxygenase activity or lipid peroxidation would produce a novel post-translational modification of proteins.

The reactivity of LGE_2 with proteins exceeds that of 4-hydroxynonenal (8). Characterization of the pyrrole-derived LG-lysine lactam as a major adduct of this amino acid in proteins provided a basis for the development of an LC/MS/MS method for its analysis by stable isotope dilution in proteolytic digests of proteins (8). Utilizing this analytical approach, we have demonstrated formation of LG-lysine adducts on COX-1 and COX-2 following the oxygenation of arachidonic acid as well as with proteins coincubated with COX and arachidonic acid (4). The reaction of LGs with the COX enzymes is rapid, essentially complete within 2 minutes after the addition of arachidonic acid as substrate. These findings formed the basis for the hypothesis that levuglandinyl adducts of proteins could be generated in cells following acute or chronic activation of COX.

LEVUGLANDINYL-LYSINE LACTAM ADDUCTS FORM IN CELLS AND TISSUES EXPRESSING COX

LG adducts in cells. To determine whether LG adducts could be formed in cells in the presence of enzymatic metabolism of PGH_2, we analyzed the formation of LG adducts of proteins during platelet activation by arachidonic acid (6). We demonstrated formation of these adducts, which is inhibited by the COX inhibitor indomethacin, and is enhanced by an inhibitor of thromboxane synthase. These data established that LG-lysine adducts are formed via a COX-dependent pathway in whole cells, even in the presence of an enzyme that metabolizes PGH_2. Formation of LG adducts of protein also following activation of platelets by thrombin indicates that a physiological stimulus is sufficient to lead to lipid modification of proteins through the levuglandin pathway.

LG adducts in tissues. To extend these observations to whole animals and because COX activity has been associated with the progression of AD, we determined the levels of LG-adducts of cerebral protein in transgenic mice that overexpress human COX-2 in brain under control of the Thy-1 promoter. In these transgenic mice, behavioral studies point to an age-related loss of cognitive function in association with neuronal apoptosis and inflammatory changes (3). We have demonstrated a marked increase in LG-lysine lactam in proteins isolated from hemispheres of these mice expressing human COX-2 compared to non-transgenic animals (Figure 2).

LEVUGLANDINS AND AMYLOID β

Levuglandins also can form intermolecular crosslinks. Importantly, the initial lysyl-levuglandin adducts themselves are also highly reactive; the Schiff base adduct with lysine can undergo nucleophilic attack (15) and the oxidation of the pyrrole adducts generates sites of electrophilic reactivity (1, 2). Thus, lysyl-LG adducts can form covalent bonds with other nucleophiles leading to intermolecular crosslinking. Such crosslinking has been demonstrated for adducts derived from synthetic levuglandin E_2. LGE_2 can avidly induce protein-protein cross-links (19), as well as protein-DNA

crosslinks (27). We have formed time-dependent cyclooxygenase-derived protein that will not enter into or migrate on polyacrylamide gels, which probably results from formation of cyclooxygenase multimers (4). The reactivity of the initial lysyl-LG adducts also is clearly demonstrated by the evidence that spermine will react covalently with COX-2 molecules that have been adducted by oxygenated products of arachidonic acid (4).

Acceleration of Aβ_{1-42} oligomerization by LGE2. Aggregation of proteins, particularly amyloid-β (Aβ), is a pathologic hallmark of AD. Since LGE_2 can crosslink proteins, we examined the effect of LGE_2 on Aβ. We demonstrated that addition of PGH_2 or LGE_2 to Aβ markedly accelerates formation of soluble oligomers that have the ultrastructural features of Aβ-derived diffusible ligands (ADDL) (7). Oligomerization of Aβ by LGE_2 occurs with ratios of LGE_2:Aβ of only 1:10. The incorporation of more Aβ molecules into the oligomers than molecules of LG indicates that crosslinking is not the sole basis for the intermolecular bonding in these oligomers. These findings invite speculation that either crosslinking to form a dimer or formation of an LG adducted monomer with altered lipophilicity could initiate or "seed" an oligomerization process (28) that proceeds via a different type of intermolecular bonding (21). Analogous mechanisms have been described for the lipid modification of Aβ by GM1 ganglioside (17) or by cholesterol-derived molecules (36).

Increased concentrations of the soluble oligomers of Aβ are present in brains of patients with AD (14). Levels of ADDLs in cerebral spinal fluid are elevated in patients with AD compared to non-demented age-matched patients (13). Soluble oligomers of Aβ have been shown to produce neurotoxicity as well as impaired neuronal function (9, 16, 23, 25, 28, 32, 33). These effects on neurons have been demonstrated with dimers and trimers of Aβ purified from brains of patients with AD, from soluble oligomers generated from Aβ in vitro and from Aβ oligomers in conditioned medium of cultured CHO cells transfected with the APP V171F mutant gene. Aβ oligomers have been found to be directly toxic to primary cultures of embryonal neurons and to cells in hippocampal brain slices. Microglial-dependent neurotoxic effects were produced in embryonal hippocampal neurons by Aβ dimers and trimers. In addition to cell toxicity, there is evidence for inhibition of hippocampal long-term potentiation in mice in vivo by oligomers of Aβ. Recently, it has been suggested that the functional disruption of specific synapses by ADDLs may constitute the molecular mechanism for memory loss in early AD (22).

Formation of LG adducts of Aβ and other proteins inhibits the catalytic function of proteasomes (10). This finding is of particular interest in the context of evidence that the proteasome exerts a neuroprotective function (11, 12), that proteasome function is impaired in AD (11), and that Aβ oligomers accumulate in the neuronal endosomes in AD (31). Lipid modification of proteins is known to alter the targeting and function of proteins, and this knowledge provides a framework for considering the potential consequences of LG adducts of proteins.

SUMMARY

Levuglandins are highly reactive products of the cyclooxygenases that form covalent adducts and crosslinks of proteins. LG adducts are present in the brains of mice that overexpress COX-2 and have age-dependent loss of cognitive function. PGH_2, via LGs, accelerates oligomerization of amyloid β, producing a species of Aβ that has been associated with neurotoxicity. A consideration of the consequences of COX activity in the brain includes this novel form of lipid modification of proteins.

ACKNOWLEDGMENTS

This work was supported by grants from the American Health Assistance Foundation, the American Federation for Aging Research, and the NIH (GM15431, AG15799). J. A. O. is the Thomas F. Frist, Sr. Prof of Medicine.

REFERENCES

1. Amarnath V, Valentine WM, Amarnath K, Eng MA, Graham DG (1994) The mechanism of nucleophilic substitution of alkylpyrroles in the presence of oxygen. *Chem Res Toxicol* 7:56-61.

2. Amarnath V, Valentine WM, Montine TJ, Patterson WH, Amarnath K, Bassett CN, Graham DG (1998) Reactions of 4-hydroxy-2(E)-nonenal and related aldehydes with proteins studied by carbon-13 nuclear magnetic resonance spectroscopy. *Chem Res Toxicol* 11:317-328.

3. Andreasson KI, Savonenko A, Vidensky S, Goellner JJ, Zhang Y, Shaffer A, Kaufmann WE, Worley PF, Isakson P, Markowska AL (2001) Age-dependent cognitive deficits and neuronal apoptosis in cyclooxygenase-2 transgenic mice. *J Neurosci* 21:8198-8209.

4. Boutaud O, Brame CJ, Chaurand P, Li J, Rowlinson SW, Crews BC, Ji C, Marnett LJ, Caprioli RM, Roberts LJ, 2nd, et al (2001) Characterization of the lysyl adducts of prostaglandin H-synthases that are derived from oxygenation of arachidonic acid. *Biochemistry* 40:6948-6955.

5. Boutaud O, Brame CJ, Salomon RG, Roberts LJ, 2nd, Oates JA (1999) Characterization of the lysyl adducts formed from prostaglandin H2 via the levuglandin pathway. *Biochemistry* 38:9389-9396.

6. Boutaud O, Li J, Zagol I, Shipp EA, Davies SS, Roberts LJ, 2nd, Oates JA (2003) Levuglandinyl adducts of proteins are formed via a prostaglandin H2 synthase-dependent pathway after platelet activation. *J Biol Chem* 278:16926-16928.

7. Boutaud O, Ou JJ, Chaurand P, Caprioli RM, Montine TJ, Oates JA (2002) Prostaglandin H2 (PGH_2) accelerates formation of amyloid β_{1-42} oligomers. *J Neurochem* 82:1003-1006.

8. Brame CJ, Salomon RG, Morrow JD, Roberts LJ, 2nd (1999) Identification of extremely reactive gamma-ketoaldehydes (isolevuglandins) as products of the isoprostane pathway and characterization of their lysyl protein adducts. *J Biol Chem* 274:13139-13146.

9. Dahlgren KN, Manelli AM, Stine WB, Jr., Baker LK, Krafft GA, LaDu MJ (2002) Oligomeric and fibrillar species of amyloid-β peptides differentially affect neuronal viability. *J Biol Chem* 277:32046-32053.

10. Davies SS, Amarnath V, Montine KS, Bernoud-Hubac N, Boutaud O, Montine TJ, Roberts LJ, 2nd (2002) Effects of reactive gamma-ketoaldehydes formed by the isoprostane pathway (isoketals) and cyclooxygenase pathway (levuglandins) on proteasome function. *FASEB J* 16:715-717.

11. Ding Q, Keller JN (2001) Proteasomes and proteasome inhibition in the central nervous system. *Free Radic Biol Med* 31:574-584.

12. Ding Q, Reinacker K, Dimayuga E, Nukala V, Drake J, Butterfield DA, Dunn JC, Martin S, Bruce-Keller AJ, Keller JN (2003) Role of the proteasome in protein oxidation and neural viability following low-level oxidative stress. *FEBS Lett* 546:228-232.

13. Georganopoulou DG, Chang L, Nam JM, Thaxton CS, Mufson EJ, Klein WL, Mirkin CA (2005) Nanoparticle-based detection in cerebral spinal fluid of a soluble pathogenic biomarker for Alzheimer's disease. *Proc Natl Acad Sci U S A* 102:2273-2276

14. Gong Y, Chang L, Viola KL, Lacor PN, Lambert MP, Finch CE, Krafft GA, Klein WL (2003) Alzheimer's disease-affected brain: Presence of oligomeric Aβ ligands (ADDLs) suggests a molecular basis for reversible memory loss. *Proc Natl Acad Sci U S A* 100:10417-10422.

15. Harada K (1970) Additions to the azomethine group. In: *The Chemistry of Carbon-Nitrogen Double Bound*, S Patai (eds). Interscience Publishers:

London - New York - Sydney - Toronto. pp. 255-298.

16. Hartley DM, Walsh DM, Ye CP, Diehl T, Vasquez S, Vassilev PM, Teplow DB, Selkoe DJ (1999) Protofibrillar intermediates of amyloid β-protein induce acute electrophysiological changes and progressive neurotoxicity in cortical neurons. *J Neurosci* 19:8876-8884.

17. Hayashi H, Kimura N, Yamaguchi H, Hasegawa K, Yokoseki T, Shibata M, Yamamoto N, Michikawa M, Yoshikawa Y, Terao K, et al (2004) A seed for Alzheimer amyloid in the brain. *J Neurosci* 24:4894-4902.

18. in t' Veld BA, Ruitenberg A, Hofman A, Launer LJ, van Duijn CM, Stijnen T, Breteler MM, Stricker BH (2001) Nonsteroidal antiinflammatory drugs and the risk of Alzheimer's disease. *N Engl J Med* 345:1515-1521.

19. Iyer RS, Ghosh S, Salomon RG (1989) Levuglandin E2 crosslinks proteins. *Prostaglandins* 37:471-480.

20. Iyer RS, Kobierski ME, Salomon RG (1994) Generation of pyrroles in the reaction of levuglandin E2 with proteins. *J Org Chem* 59:6038-6043.

21. Koppaka V, Paul C, Murray IV, Axelsen PH (2003) Early synergy between $A\beta_{42}$ and oxidatively damaged membranes in promoting amyloid fibril formation by $A\beta_{40}$. *J Biol Chem* 278:36277-36284.

22. Lacor PN, Buniel MC, Chang L, Fernandez SJ, Gong Y, Viola KL, Lambert MP, Velasco PT, Bigio EH, Finch CE, et al (2004) Synaptic targeting by Alzheimer's-related amyloid β oligomers. *J Neurosci* 24:10191-10200.

23. Lambert MP, Barlow AK, Chromy BA, Edwards C, Freed R, Liosatos M, Morgan TE, Rozovsky I, Trommer B, Viola KL, et al (1998) Diffusible, nonfibrillar ligands derived from $A\beta_{1-42}$ are potent central nervous system neurotoxins. *Proc Natl Acad Sci U S A* 95:6448-6453.

24. Lim GP, Yang F, Chu T, Chen P, Beech W, Teter B, Tran T, Ubeda O, Ashe KH, Frautschy SA, et al (2000) Ibuprofen suppresses plaque pathology and inflammation in a mouse model for Alzheimer's disease. *J Neurosci* 20:5709-5714.

25. Michikawa M, Gong JS, Fan QW, Sawamura N, Yanagisawa K (2001) A novel action of Alzheimer's amyloid β-protein (Aβ): oligomeric Aβ promotes lipid release. *J Neurosci* 21:7226-7235.

26. Montine TJ, Sidell KR, Crews BC, Markesbery WR, Marnett LJ, Roberts LJ, 2nd, Morrow JD (1999) Elevated CSF prostaglandin E_2 levels in patients with probable AD. *Neurology* 53:1495-1498.

27. Murthi KK, Friedman LR, Oleinick NL, Salomon RG (1993) Formation of DNA-protein cross-links in mammalian cells by levuglandin E2. *Biochemistry* 32:4090-4097.

28. Roher AE, Chaney MO, Kuo YM, Webster SD, Stine WB, Haverkamp LJ, Woods AS, Cotter RJ, Tuohy JM, Krafft GA, et al (1996) Morphology and toxicity of $A\beta_{1-42}$ dimer derived from neuritic and vascular amyloid deposits of Alzheimer's disease. *J Biol Chem* 271:20631-20635.

29. Salomon RG, Miller DB, Zagorski MG, Coughlin DJ (1984) Solvent-induced fragmentation of prostaglandin endoperoxides. New aldehyde products from PGH_2 and a novel intramolecular 1,2-hydride shift during endoperoxide fragmentation in aqueous solution. *J Am Chem Soc* 106:6049-6060.

30. Stewart WF, Kawas C, Corrada M, Metter EJ (1997) Risk of Alzheimer's disease and duration of NSAID use. *Neurology* 48:626-632.

31. Takahashi RH, Almeida CG, Kearney PF, Yu F, Lin MT, Milner TA, Gouras GK (2004) Oligomerization of Alzheimer's β-amyloid within processes and synapses of cultured neurons and brain. *J Neurosci* 24:3592-3599.

32. Tong L, Thornton PL, Balazs R, Cotman CW (2001) $\beta\text{-amyloid}_{1-42}$ impairs activity-dependent cAMP-response element-binding protein signaling in neurons at concentrations in which cell survival is not compromised. *J Biol Chem* 276:17301-17306.

33. Walsh DM, Klyubin I, Fadeeva JV, Cullen WK, Anwyl R, Wolfe MS, Rowan MJ, Selkoe DJ (2002) Naturally secreted oligomers of amyloid β protein potently inhibit hippocampal long-term potentiation in vivo. *Nature* 416:535-539.

34. Xiang Z, Ho L, Yemul S, Zhao Z, Qing W, Pompl P, Kelley K, Dang A, Teplow D, Pasinetti GM (2002) Cyclooxygenase-2 promotes amyloid plaque deposition in a mouse model of Alzheimer's disease neuropathology. *Gene Expr* 10:271-278.

35. Zagol-Ikapitte I, Bernoud-Hubac N, Amarnath V, Roberts LJ, 2nd, Boutaud O, Oates JA (2004) Characterization of Bis(levuglandinyl) Urea Derivatives as Products of the Reaction between Prostaglandin H_2 and Arginine. *Biochemistry* 43:5503-5510.

36. Zhang Q, Powers ET, Nieva J, Huff ME, Dendle MA, Bieschke J, Glabe CG, Eschenmoser A, Wentworth P, Jr., Lerner RA, et al (2004) Metabolite-initiated protein misfolding may trigger Alzheimer's disease. *Proc Natl Acad Sci U S A* 101:4752-4757.