



Inhibition of A β production by NF- κ B inhibitors

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Received 26 April 2006; received in revised form 9 October 2006; accepted 21 December 2006

Abstract

The transcription factor nuclear factor κ B (NF- κ B) is widely expressed in the nervous system and increased NF- κ B immunoreactivity has been observed in Alzheimer's disease (AD) brains in the nuclei of neurons within the vicinity of diffuse β -amyloid plaques. β -Amyloid (A β) peptides are the main constituent of senile plaques and are known to stimulate NF- κ B activity. In the present study, we investigated the effect of various NF- κ B inhibitors on the production of A β_{1-40} , A β_{1-42} , secreted APP (sAPP β and sAPP α) and APP C-terminal fragments (APP-CTF) using CHO cells overexpressing the β -amyloid precursor protein (APP). Our data show that NF- κ B inhibitors decrease both A β_{1-40} and A β_{1-42} production. In addition, we show that some NF- κ B inhibitors decrease sAPP β and APP-CTF β suggesting that they reduce the β -secretase cleavage of APP. Altogether our data suggest that NF- κ B inhibitors may be of therapeutic importance for the treatment of AD pathology not only by blocking inflammatory processes but also by directly inhibiting the production of A β peptides.

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Keywords: Alzheimer; APP processing; NF- κ B; Amyloid

The brains of Alzheimer's disease (AD) patients are characterized by the accumulation of a 38–43-amino acids peptide termed amyloid β -peptide or A β [16]. This peptide is the major component of senile plaques found in AD and is proposed as a key player in the pathobiology of AD since all familial forms of the disease are associated with an increased A β accumulation. A β is a proteolytic fragment of the large amyloid precursor protein (APP) [6]. It is cleaved by β -secretase generating secreted APP β (sAPP β) and the carboxyl terminal intracellular fragment (APP-CTF β) which is further cleaved by γ -secretase to produce A β peptides [17], whereas the α -secretase cleavage of APP takes place within the A β sequence precluding A β production, leading to the secretion of sAPP α and the accumulation of membrane-coupled APP CTF- α . The γ -secretase cleavage is not precise and leads to the production of a series of A β peptides of 38–43 amino acids. A β_{1-42} is more fibrillogenic than shorter A β peptides but a higher proportion of A β is produced as A β_{1-40} compared to A β_{1-42} . Elevated A β_{1-42} concentrations are thought to drive the formation of insoluble fibrils resulting in the deposition of A β as amyloid plaques. For this reason A β_{1-42} is considered to have

a causative role in the etiology of AD and therapies particularly targeting A β_{1-42} are expected to have the greatest impact on β -amyloid pathology.

The transcription factor NF- κ B is widely expressed in the nervous system and particularly in synaptic terminals. In non-stimulated cells, NF- κ B is sequestered in the cytoplasm by inhibitory units called I κ B proteins. Stimulation of cells by various inducers causes I κ B phosphorylation and its subsequent degradation by the proteasome [21]. Liberated NF- κ B is transported in the nucleus, where it induces transcription of target genes, including I κ B as an autoregulatory loop [21].

Studies of postmortem brain tissue from patients with AD have revealed increased NF- κ B immunoreactivity in neurons and astrocytes in the immediate vicinity of β -amyloid plaques [7,19]. Many genes newly induced in AD are under immediate-early transcriptional control of NF- κ B [11] also suggesting that NF- κ B pathway is activated in AD brains. Other studies have shown that A β peptides can activate NF- κ B in primary neurons and astrocytes [2] suggesting a molecular mechanism by which A β may act during AD pathogenesis. Recently, it has been reported that indomethacin (a non steroidal anti-inflammatory drug) can lead to a reduction in the level of A β peptides and NF- κ B in the brains of a transgenic mouse model of AD (Tg2576) whereas another anti-inflammatory compound, nimesulide had

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no effect on either A β peptides or NF- κ B [18] suggesting that NF- κ B activation blockade could reduce the amyloid pathology in Tg2576 mice. Since A β itself is known to induce NF- κ B, the reduced NF- κ B activity observed in Tg2576 mice treated with indomethacin could alternatively be secondary to the reduction of A β accumulation observed. We therefore investigated the effect of various NF- κ B inhibitors on the production of A β peptides using Chinese Hamster Ovary cells stably transfected with wildtype APP 751 (7W CHO), overproducing human A β . The effect of NF- κ B inhibitors was also tested on the accumulation of APP C-terminal fragments (APP-CTF) in order to determine whether NF- κ B inhibitors could impact γ -secretase activity. Additionally, we investigated the effect of NF- κ B inhibitors on the secretion of sAPP α and sAPP β to determine a possible effect of NF- κ B inhibitors on α -secretase or β -secretase activities.

For this study, we tested the effect of different NF- κ B inhibitors (NF- κ B SN50, parthenolide, hypostoxide, capsaicin, andrographolide, Caffeic Acid Phenethyl Ester (CAPE), artemisinin, celastrol, 6-amino-4-(4-phenoxyphenyl-ethyl-amino)quinazoline (quinazoline), isohelenin, kamebakaurin) on the production of A β _{1–40} and A β _{1–42} peptides by CHO cells overexpressing APP. NF- κ B SN50 is a selective, cell permeable NF- κ B inhibitor peptide which contains the nuclear localization sequence (NLS) of the transcription factor NF- κ B p50 linked to the hydrophobic region (h-region) of the signal peptide of Kaposi fibroblast growth factor (K-FGF). The N-terminal K-FGF h-region confers cell-permeability, while the NLS inhibits translocation of the NF- κ B active complex into the nucleus [14]. Parthenolide and isohelenin are two sesquiterpene lactone known to block NF- κ B activation [3]. Andrographolide is a bicyclic diterpenoid lactone, which inhibits NF- κ B binding to DNA [8]. CAPE is an active component of propolis from honeybee hives that prevents the translocation of the p65 subunit of NF- κ B to the nucleus [15]. Artemisinin (a potent anti-malarial sesquiterpene) and celastrol (sesquiterpene ester) have been shown to block NF- κ B activation [1,10]. Kamebakaurin is a kaurane diterpene, which prevents the DNA binding activity of activated NF- κ B but does not block its nuclear translocation [13]. Quinazoline is a low molecular weight inhibitor of NF- κ B transcriptional activation [20].

Briefly, 7W CHO cells were maintained in DMEM (ATCC, Manassas, VA, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 1 \times Penicillin–Streptomycin Fungizone mixture (Cambrex, Rockland, ME, USA) and 0.3% Geneticin (Invitrogen, Carlsbad, CA, USA). Cells were plated on 96 well-culture plates at a density of 5 \times 10⁴ cells per well in 200 μ L of culture medium. All the inhibitors used in this study were purchased from EMD Biosciences Inc. (San Diego, CA, USA). The concentration of inhibitors used to achieve NF- κ B inhibition were chosen according to referenced publications. For each inhibitor, experiments were performed in quadruplicate and A β _{1–40} and A β _{1–42} levels were evaluated following 18 h of incubation using ELISA kits (Invitrogen Biosource Inc., Carlsbad, CA, USA) according to the recommendations of the manufacturer. For the measurement of A β _{1–40}, samples were diluted 20-fold with the sample diluent provided in the kits whereas for A β _{1–42} determination,

samples were diluted only two-fold. Results were expressed as the percentage of A β _{1–40} or A β _{1–42} measured in control samples. No direct toxicity (monitored by lactate-dehydrogenase release in the culture medium) was observed for the doses of NF- κ B inhibitors tested (data not shown). Interestingly, the different NF- κ B inhibitors tested appear to diminish A β production with different potency and show a differential effect towards A β _{1–40} and A β _{1–42} production (Fig. 1A). For instance, NF- κ B SN50 at 20 μ M inhibits A β _{1–42} (~40% inhibition) more potently than A β _{1–40} (~9%). Similarly, hypostoxide displays more potency towards A β _{1–42} than A β _{1–40} inhibition. Quinazoline, artemisinin (at 1 μ M) and isohelenin (at 5 μ M) inhibit A β _{1–40} secretion more potently than A β _{1–42} whereas the other compounds tested display analogous effects on A β _{1–40} and A β _{1–42} production.

NF- κ B activation can be blocked indirectly by inhibiting I kappa B kinase (IKK-2). We therefore tested a cell-permeable ureidocarboxamido thiophene compound that acts as a potent inhibitor of IKK-2 ([5-(*p*-Fluorophenyl)-2-ureido]thiophene-3-carboxamide or IKK-2 inhibitor IV). IKK-2 inhibitor IV also appears to significantly inhibit both A β _{1–40} and A β _{1–42} production (Fig. 1A). These data support the concept that the secretion and/or production of A β _{1–40} and A β _{1–42} is NF- κ B dependent.

In order to verify that NF- κ B inhibitors were impacting the secretion of A β , we measured the intracellular level of A β _{1–40}. Briefly, 7W CHO were treated with different NF- κ B inhibitors (Fig. 1B). Following 18 h of incubation, cells were washed with PBS and lysed with 100 μ L of ice-cold M-PER[®] Reagent (Pierce, IL, USA) containing 1 mM phenylmethanesulfonyl fluoride and 1 mM sodium orthovanadate. Cellular lysates were diluted five-fold with the standard diluent provided in the A β _{1–40} ELISA kit (Biosource, CA) before intracellular A β _{1–40} quantification. Data show that intracellular A β _{1–40} represent only a small fraction of the A β _{1–40} produced by the cells (approximately 1.5% of the amount of A β _{1–40} secreted in the culture medium). Interestingly, among the NF- κ B inhibitors tested, only CAPE, celastrol and kamebakaurin were able to significantly lower intracellular A β _{1–40}, but no intracellular accumulation of A β was observed with any of the compounds suggesting that the secretion of A β is not impacted by NF- κ B inhibition (Fig. 1B).

In order to verify that NF- κ B inhibition was achieved, we measured the production of prostaglandin E₂ (PGE₂), a main product of cyclooxygenase-2 (whose expression is regulated by NF- κ B) employing the same culture paradigm used to measure A β production. This was necessarily an indirect way of assessing NF- κ B inhibition as the inhibitors are purported to achieve their effects by different mechanisms (some inhibit nuclear translocation of NF- κ B, while others inhibit its binding to DNA). We analyzed conditioned cell culture media for PGE₂ (a main metabolite of cyclooxygenase-2, expression of which is NF- κ B regulated), using a commercially available ELISA according to the strict recommendations of the manufacturer (Cayman Chemical, Ann Arbor, MI, USA), following treatment of 7W CHO cells with various NF- κ B inhibitors for 18 h. Our results (Fig. 2) demonstrate a differential inhibition of 7W CHO cell PGE₂ production showing that the NF- κ B inhibitors tested inhibit NF- κ B activity with different potencies [4]. Interestingly, we found

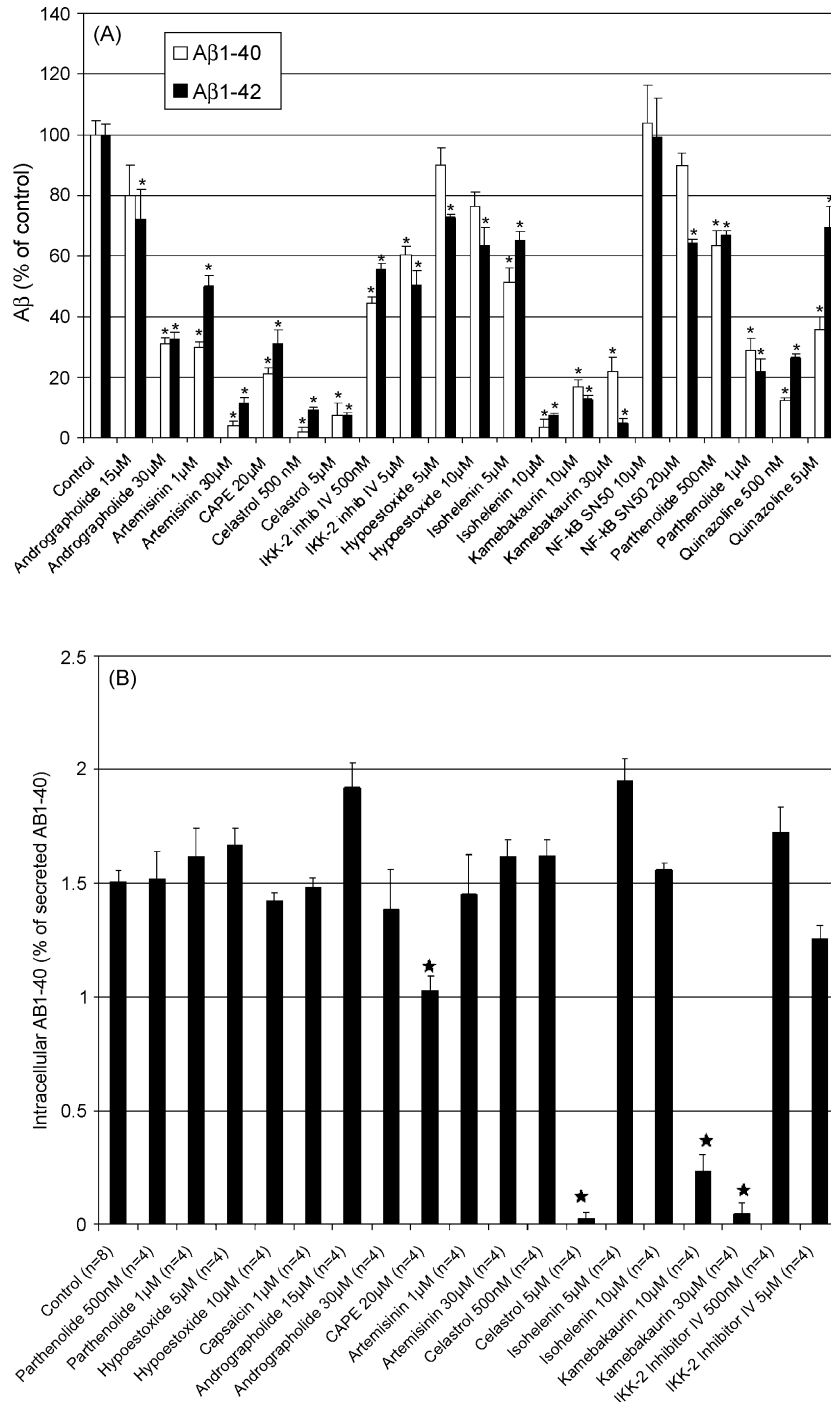


Fig. 1. (A) Analysis of Aβ by ELISA from 7W CHO cells treated with different NF-κB inhibitors. Aβ₁₋₄₀ and Aβ₁₋₄₂ levels were normalized to values obtained from vehicle-treated cells and expressed as percentage of Aβ₁₋₄₀ or Aβ₁₋₄₂ values obtained in the control conditions. The bar graph represents the effect of NF-κB inhibitors on Aβ₁₋₄₀ and Aβ₁₋₄₂ production. ANOVA revealed significant main effects ($P < 0.05$) for the different treatment conditions as well as significant dose dependent effects of the compounds on Aβ₁₋₄₀ and Aβ₁₋₄₂ production ($P < 0.05$). The symbol (*) on the bar graph denotes significant differences ($P < 0.05$) revealed by post hoc analysis between the value of Aβ₁₋₄₀ or Aβ₁₋₄₂ obtained in the control and treated conditions. (B) Effect of NF-κB inhibitors on intracellular Aβ₁₋₄₀ levels in 7W CHO cells. Aβ₁₋₄₀ levels were normalized to Aβ₁₋₄₀ values obtained from vehicle-treated cells. The symbol (*) on the bar graph denotes significant differences ($P < 0.05$) revealed by post hoc analysis between the value of Aβ₁₋₄₀ obtained in the control and treated conditions.

178 a correlation between the amount of PGE₂ inhibition and the
 179 amount of Aβ inhibition (data not shown) suggesting that the
 180 amount of Aβ inhibition observed is proportional to the extent of
 181 NF-κB pathway inhibition. However, some compounds display
 182 preferential inhibitory effects towards Aβ₁₋₄₀ or Aβ₁₋₄₂ sug-

gesting that this selectivity towards the inhibition of a particular
 Aβ species may rely on additional properties of the molecules
 tested independently of their effect on NF-κB pathway.

In addition, we tested the effect of NF-κB inhibitors and
 of the IKK-2 inhibitor IV on Aβ₁₋₄₀ and Aβ₁₋₄₂ secretion

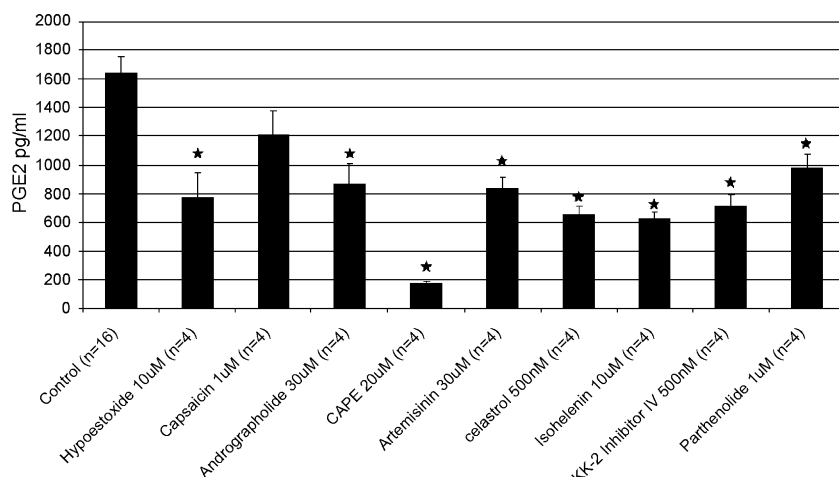


Fig. 2. Analysis of PGE₂ production by ELISA from cultured cells treated with different NF-κB inhibitors. ANOVA revealed significant main effects ($P < 0.05$) for the different treatment conditions. The symbol (*) on the bar graph denotes significant differences ($P < 0.05$) revealed by post hoc analysis between the value of PGE₂ obtained in the control and treated conditions.

188 by human neuroblastoma SH-SY5Y cells (ATCC, Manas-
 189 sas, VA, USA). SH-SY5Y cells were grown in Ham's F12
 190 medium supplemented with 10% fetal bovine serum and 1×
 191 Penicillin–Streptomycin Fungizone mixture. Confluent SH-
 192 SY5Y cells were treated for 48 h with celastrol, CAPE,
 193 artemisinin and the IKK-2 inhibitor IV. Culture media was then
 194 collected and evaluated for Aβ_{1–40} as previously described and
 195 Aβ_{1–42} using a high sensitivity Aβ_{1–42} ELISA (detection limit
 196 <1 pg/ml, Invitrogen Biosource Inc., Carlsbad, CA, USA). Data
 197 reveal that CAPE, celastrol, artemisinin and the IKK-2 Inhibitor
 198 IV significantly reduce Aβ_{1–40} and Aβ_{1–42} production by SH-
 199 SY5Y cells confirming that NF-κB also regulates Aβ production
 200 in neuronal like cells (Fig. 3).

201 We next tested the effect of NF-κB inhibitors on the accu-
 202 mulation of intracellular APP-CTF fragments by western-blot,
 203 using 7W CHO cells, to determine whether NF-κB inhibitors
 204 can affect APP processing. Briefly, 7W CHO cells were plated
 205 onto 24-well cell culture plates. Confluent 7W CHO cells
 206 were treated for 18 h with different NF-κB inhibitors. Cellular
 207 proteins were extracted with 150 μL of ice-cold M-PER®
 208 Reagent (Pierce Biotechnology, Rockford, IL, USA) contain-
 209 ing 1 mM phenylmethanesulfonyl fluoride and 1 mM sodium
 210 orthovanadate. Samples were sonicated, denatured by boiling
 211 in Laemmli buffer (Bio-Rad, Hercules, CA, USA) and resolved
 212 onto 4–20% gradient polyacrylamide gels (Bio-Rad, Hercules,
 213 CA, USA). After electrotransferring onto polyvinylidene diflu-
 214 oride membranes, Western-blot were immunoprobed with an
 215 anti-APP C-terminal (751–770) antibody (EMD Biosciences
 216 Inc., San Diego, CA, USA), with the antibody 22C11 (Chem-
 217 icon, Temecula, CA, USA) that recognizes full length APP and
 218 with an anti-actin antibody (Chemicon, Temecula, CA, USA)
 219 used as a reference antibody to quantify the amount of pro-
 220 teins electrotransferred. Additionally, sAPPα was detected by
 221 Western-blot in the culture medium surrounding 7W CHO cells
 222 using the antibody 6E10 (Signet Laboratories Inc., Dedham,
 223 MA, USA) which recognizes the amino-acids 1–17 of Aβ and
 224 sAPPβ was detected in the culture medium using an anti-human
 225 sAPPβ antibody (Immuno-Biological Laboratories Co. Ltd.,

226 Gunma, Japan). APP CTF/Actin, sAPPα/(sAPPα + sAPPβ) and
 227 sAPPβ/(sAPPα + sAPPβ) signals intensity ratios were quan-
 228 tified by chemoluminescence imaging with the ChemiDoc™
 229 XRS (Bio-Rad, Hercules, CA). Our data show that the differ-
 230 ent NF-κB inhibitors tested do not induce the accumulation of
 231 APP CTF (Fig. 4A) suggesting that NF-κB inhibitors lower Aβ
 232 production without inhibiting γ-secretase activity (an inhibition
 233 of γ-secretase activity would lead to a significant accumulation
 234 of APP CTFα and CTFβ). Celastrol, kamebakaurin appear to
 235 stimulate the ratio of sAPPα/(sAPPα + sAPPβ) and to decrease
 236 the ratio of sAPPβ/(sAPPα + sAPPβ), as well as APP CTFβ
 237 production (Fig. 4B) suggesting that they inhibit the β-cleavage
 238 of APP.

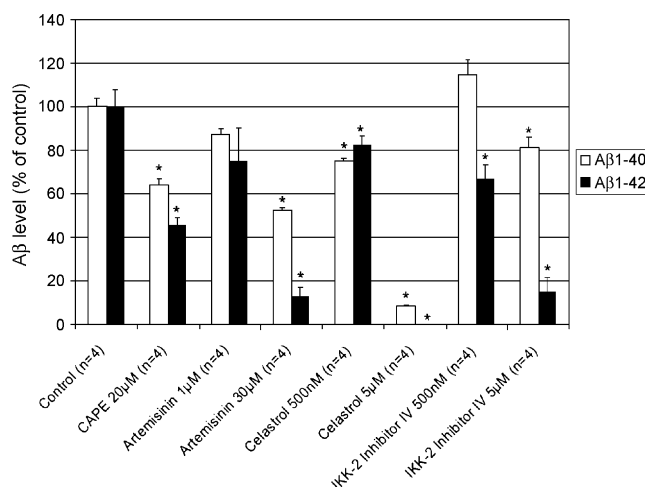


Fig. 3. Analysis of Aβ by ELISA from human neuroblastoma SH-SY5Y cells treated with different NF-κB inhibitors for 48 h. Aβ_{1–40} and Aβ_{1–42} levels were normalized to values obtained from vehicle-treated cells and expressed as percentage of Aβ_{1–40} or Aβ_{1–42} values obtained in the control conditions. The bar graph represents the effect of NF-κB inhibitors on Aβ_{1–40} and Aβ_{1–42} production. ANOVA revealed significant main effects ($P < 0.05$) for the different treatment conditions as well as significant dose dependent effects of the compounds on Aβ_{1–40} and Aβ_{1–42} production ($P < 0.05$). The symbol (*) on the bar graph denotes significant differences ($P < 0.05$) revealed by post hoc analysis between the value of Aβ_{1–40} or Aβ_{1–42} obtained in the control and treated conditions.

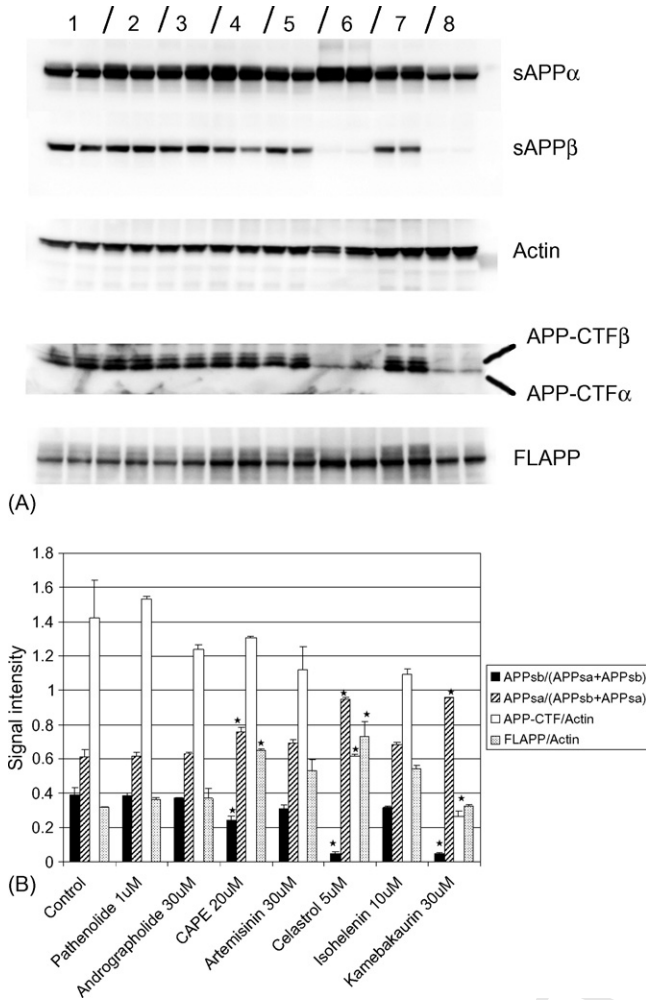


Fig. 4. Analysis of APP processing after treatment of CHO cells with different NF- κ B inhibitors. (A) Lanes 1: control; 2: parthenolide 1 μ M; 3: andrographolide 30 μ M; 4: CAPE 20 μ M; 5: artemisinin 30 μ M; 6: celestrol 5 μ M; 7: isohelenin 10 μ M; 8: kamebakaurin 30 μ M. (B) The chemoluminescent signal intensities of the ratios of sAPP α /(sAPP α + sAPP β), sAPP β /(sAPP α + sAPP β), full length APP (FL APP)/actin and APP-CTF α + APP-CTF β (APP CTF)/actin are represented on the histogram. The symbol (*) on the bar graph denotes significant differences ($P < 0.05$) revealed by post hoc analysis between the value of the chemoluminescent signals obtained in the control and treated conditions. Data analysis revealed that CAPE, celestrol and kamebakaurin significantly decreased sAPP β and APP-CTF but increased sAPP α production. Full length APP increment was observed following CAPE and celestrol treatment.

As several other non steroidal anti-inflammatory drugs (NSAIDs) can also inhibit NF- κ B activation (such as sulindac sulphide or R-flurbiprofen), it remains possible that selective NSAIDs presenting an inhibitory activity towards NF- κ B will be able to lower A β levels independently of their inhibitory action on cyclooxygenase activities. However, NSAIDs lower A β ₁₋₄₂ with low efficiency (IC₅₀ generally greater than 50 μ M) [22] and some cyclooxygenase-2 selective NSAIDs have been shown to raise A β ₁₋₄₂ levels by 200% [12]. Unfortunately also to date, clinical trials designed to inhibit cyclooxygenase activity with NSAIDs have failed to demonstrate efficacy in the treatment of AD patients [9]. Among the NF- κ B inhibitors that we tested, celestrol appears the most potent (more than 90% inhibition of A β ₁₋₄₀ and A β ₁₋₄₂ with 500 nM in 7W CHO cells). Acute

treatment with celestrol in rodent has revealed that this compound can decrease brain NF- κ B immunostaining, astrogliosis and neurodegeneration in mouse models of Parkinson's disease and Huntington's disease [5]. Neuroinflammation is a prominent feature of AD pathology and NF- κ B blockade is also expected to be beneficial by decreasing astrogliosis and microgliosis in addition to reducing A β accumulation as outlined in this manuscript. However, NF- κ B influences the expression of a complex array of genes in the nervous system and the consequences of a chronic down regulation of the NF- κ B pathway are not well established. It is therefore premature to consider NF- κ B as a viable therapeutic target for AD. Further work will be required to determine whether NF- κ B inhibitors can lower A β levels and improve cognition in transgenic animal models of AD and whether they can be safely used in humans.

Acknowledgments

We thank Dr. Michael Wolfe (Center for Neurologic Diseases, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA) for providing the 7W CHO cells used in this study. The authors are grateful to Mr. and Mrs. Roskamp for their generous support which helped to make this work possible.

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