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ARTICLE

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Increased NF-κB signalling up-regulates BACE1 expression and its therapeutic potential in Alzheimer's disease

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Abstract

Elevated levels of β-site APP cleaving enzyme 1 (BACE1) were found in the brain of some sporadic Alzheimer's disease (AD) patients; however, the underlying mechanism is unknown. BACE1 cleaves β-amyloid precursor protein (APP) to generate amyloid β protein (Aβ), a central component of neuritic plaques in AD brains. Nuclear factor-kappa B (NF-κB) signalling plays an important role in gene regulation and is implicated in inflammation, oxidative stress and apoptosis. In this report we found that both BACE1 and NF-κB p65 levels were significantly increased in the brains of AD patients. Two functional NF-κB-binding elements were identified in the human *BACE1* promoter region. We found that NF-κB p65 expression resulted in increased *BACE1* promoter activity and *BACE1* transcription, while disruption of NF-κB p65 decreased *BACE1* gene expression in p65 knockout (RelA-knockout) cells. In addition, NF-κB p65 expression leads to up-regulated β-secretase cleavage and Aβ production, while non-steroidal anti-inflammatory drugs (NSAIDs) inhibited *BACE1* transcriptional activation induced by strong NF-κB activator tumour necrosis factor-alpha (TNF-α). Taken together, our results clearly demonstrate that NF-κB signalling facilitates *BACE1* gene expression and APP processing, and increased BACE1 expression mediated by NF-κB signalling in the brain could be one of the novel molecular mechanisms underlying the development of AD in some sporadic cases. Furthermore, NSAIDs could block the inflammation-induced *BACE1* transcription and Aβ production. Our study suggests that inhibition of NF-κB-mediated *BACE1* expression may be a valuable drug target for AD therapy.

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Introduction

Alzheimer's disease (AD) is the most prevalent form of neurodegenerative disease leading to dementia in the aged population. There are two forms of AD: familial and sporadic. Less than 5% of all AD cases are familial, while the remaining AD cases are predominantly late-onset sporadic, and are neuropathologically and clinically indistinguishable from familial forms of AD. One of the hallmarks of AD is neuritic plaques, of which filamentous extracellular Aβ aggregates are the central component. Aβ is generated by sequential proteolytic cleavage of β-amyloid precursor protein (APP) by β-secretase and γ-secretase. APP is first cleaved by β-secretase to generate a 99-amino-acid membrane-bound c-terminal fragment (C99) and C99 is subsequently cleaved by γ-secretase

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to generate Aβ. β-site APP cleaving enzyme (BACE1) is the β-secretase *in vivo*.

AD pathogenesis is believed to be multifactorial, and abnormal gene regulation could be one factor associated with abnormal processing of APP to increase Aβ level in AD. *BACE1* expression is tightly regulated at the transcription (Christensen *et al*. 2004; Li *et al*. 2006; Sun *et al*. 2005) and translation level (De Pietri Tonelli *et al*. 2004; Lammich *et al*. 2004; Rogers *et al*. 2004; Zhou & Song, 2006). Dysregulation of *BACE1* expression at transcription and translation levels may play an important role in AD pathogenesis. At transcriptional level, *BACE1* gene expression is regulated by hypoxia, and hypoxia treatment has been shown to markedly increase Aβ deposition and potentiate memory deficits in AD transgenic mice (Sun *et al*. 2006*a*). During cerebral ischaemia, BACE levels are increased in AD brains (Tesco *et al*. 2007). Furthermore, p25/cdk5 increases the amyloidogenic processing of APP through STAT3-mediated transcriptional control of BACE1 (Wen *et al*. 2008). BACE1 mRNA levels tend to increase as miR-107 levels decrease in the progression of AD (Wang *et al*. 2008). Although, in transgenic mice, there was no significant change in BACE1 mRNA level, BACE1 levels were elevated in neurons around amyloid plaques (Zhao *et al*. 2007).

NF-κB signalling plays an important role in gene regulation and is implicated in inflammation, oxidative stress, and apoptosis (Baeuerle & Henkel, 1994; Schreck *et al*. 1992; Stockley & O'Neill, 2007). All proteins of the NF-κB family share a Rel homology domain in their N-terminus. A subfamily of NF-κB proteins includes NF-κB1, NF-κB2, RelA, RelB and c-Rel. *NF-κB1* and *NF-κB2* encode for large precursor proteins p105 and p100, and subsequently p105 and p100 are processed to generate the mature NF-κB subunits p50 and p52 by the ubiquitin proteasome pathway, respectively. *RelA* gene encodes for p65 protein. NF-κB consists of a homo- or heterodimeric complex formed by RelA(p65), RelB, c-Rel, p50, and p52 and the most abundant form of the NF-κB family is the heterodimeric p65–p50 complex. Binding of inhibitor of κB (IκB) to NF-κB dimers, mostly RelA (p65)/p50, causes the dimers to be sequestered in cytoplasm and to remain inactive. When NF-κB-activating stimuli activate the IκB kinase (IKK) complex, IKK phosphorylates IκB, and NF-κB is released to the nucleus, where it interacts with NF-κB-binding elements in the promoter of target genes to modulate gene expression. In the promoter region of NF-κB-targeted genes, p65/p50 dimers bind to the sequence 5′-GGGRNNYYCC (Baldwin, 1996; Miyamoto & Verma, 1995). NF-κB can be activated by various stimuli, such as oxidative stress, mitogens, apoptotic mediators, and bacterial products (Ghosh *et al*. 1998; Siebenlist *et al*. 1994). Inflammation is one of major pathological changes in AD brains and NF-κB signalling plays an important role in inflammation and oxidative stress (Tong *et al*. 2005). NF-κB activation was implicated in H_2O_2-induced oxidative stress and NF-κB activity was increased in AD brain (Kaltschmidt *et al*. 1997; Schreck *et al*. 1991). In the present study, we found that both NF-κB and *BACE1* levels significantly increase in AD patients, and NF-κB signalling regulates *BACE1* gene expression and APP processing, indicating that NF-κB signalling plays an important role in AD pathogenesis and could be a potential drug target for AD therapy.

Materials and methods

Post-mortem brain tissues

Brain tissues from AD patients were obtained from Department of Pathology, Columbia University and University of Maryland Brain and Tissue Bank for Developmental Disorders. The cerebral cortical tissues of the frontal cortex from the post-mortem brains of 18 AD patients and 13 controls were used in this study. The age (mean $\pm$ S.D.) of AD patients was 74.89 ± 8.48 yr and for controls was 68.69 ± 10.09 yr ($p > 0.05$). There was no significant difference in post-mortem intervals between AD (13.17 ± 7.97 h) and controls (18.46 ± 6.20 h) ($p > 0.05$).

Generation of human BACE1 gene promoter constructs and other plasmids

Previously, we have constructed three 5′ upstream fragments of the human BACE1 gene amplified by PCR in front of the firefly luciferase gene of pGL3-basic vector (Christensen *et al*. 2004). Plasmid pB1P-N1, pB1P-N2 and pB1P-N3 contain the human BACE1 promoter region from -1466 to $+292$, -932 to $+292$ and -757 to $+292$, respectively. The human BACE1 promoter region -145 to $+292$ was amplified by PCR using primers -145*Xho*I (5′-ccgctcgagcctagatgtccctcc-aa) and $+292$r*Hin*dIII (5′-cacaagcttccaccataatccagctcg) and pB1P-N3 as template, and the fragment was cloned into pGL3-basic vector at *Xho*I and *Hin*dIII to generate pB1P-N4 reporter plasmid. In order to construct pBACE1-4NF-κB, double-stranded oligonucleotide 4NF-κB was generated by the oligonucleotide primers 4NF-κBf (5′-ctagcgtgaaaccccggtgaaatc-ccacgaagattcccttgtggaacccc) and 4NF-κBr (5′-tcgag-gggttccacaagggaatcttcgtgggatttcaccggggtttcacg). This

oligonucleotide consists of four putative NF-κB p65-binding elements in the human BACE1 promoter region from −1466 to +292 and was inserted in front of the firefly luciferase gene of the pGL-pL plasmid (Cai *et al*. 2008) at *Nhe*I and *Xho*I. To construct the BACE1 promoter fragment containing the mutation of the first NF-κB-binding element, PCR reactions were performed with template pB1P-G and two sets of primers BACE1U-1466 (5′-gctagctagctttccaacatatataac-3′)/NF1mr(5′-gAACtttGaAcgtgttagccaagatggt) and NF1mf (5′-acacgTtCaaaGTTcgtctctactaaaaat)/BACE1U292 (5′-cacaagcttccaccataatccagctcg). The resulting PCR fragments were annealed and served as the template in a PCR reaction with BACE1U-1466/ BACE1U292 primers to generate a mutation in the first NF-κB-binding element of the BACE1 promoter fragment. The second-round PCR product was cloned in front of the firefly luciferase gene of pGL3-basic vector at *Nhe*I and *Hin*dIII to generate plasmid pB1P-κ1m. The BACE1 promoter fragment containing the mutation of the fourth NF-κB-binding site was generated by a similar method with primers NF4mf (5′-attgtTTCTaaGGTTactgcggcaggaatcac) and NF4mr (5′-gtAACCttAGAAacaatacgatgtggca) to generate plasmid pB1P-κ4 m. The plasmids were further confirmed by DNA sequencing.

Cell culture and drug treatment

HEK293 (human embryonic kidney), N2a (Mouse neuroblastoma), SH-SY5Y (human neuroblastoma) and NIH-3T3 (mouse fibroblast) cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). SAS1 cells, a Swedish mutant APP695 stably transfected SH-SY5Y cell line (Sun *et al*. 2006*a*), were cultured in DMEM supplemented with 10% FBS and 100 μg/ml zeocin (Invitrogen, USA). 20E2 cells, HEK293 cells stably expressing Swedish mutant APP695 (Qing *et al*. 2004) were cultured in DMEM supplemented with 10% FBS and 50 μg/ml geneticin. RelA knockout (RelA-KO) fibroblast cell line, derived from E12.5–E14.5 mouse embryo fibroblasts, was cultured in DMEM supplemented with 15% FBS, β-mercaptoethanol, and ESGRO® (LIF) (Chemicon ESG1106) (Gapuzan *et al*. 2005). All cells were cultured at 37 °C in a humidified incubator containing 5% CO_2. For non-steroidal anti-inflammatory drug (NSAID) treatment, aspirin (250 mM, Sigma, USA), indomethacin (5 mM, Biomol, Germany) and (S)-ibuprofen (250 mM, Biomol) were dissolved in ethanol. The medium was changed 24 h after transfection and replaced with a medium containing 10 ng/ml TNF-α, 2.5 mM aspirin, 250 μM (S)-ibuprofen and 50 μM indomethacin for an additional 24 h.

Transfection and luciferase assay

Cells were grown to approximately 70% confluence and transfected with 2 μg plasmid DNA on a 35-mm-diameter plate for Western blot analysis and RNA extraction or 500 ng plasmid DNA on a well of a 24-well plate for luciferase assay, respectively. pCMV-Rluc (Promega, USA) was co-transfected for normalizing transfection efficiency. Transfections were performed using Lipofectamine2000 reagent (Invitrogen) according to the manufacturer's protocol. For luciferase assay, cells were harvested 24 h after transfection and lysed in 100 μl of 1× passive lysis buffer (Promega) for luciferase activity assay. The assays for firefly luciferase activity and Renilla luciferase activity were performed sequentially using one reaction tube with a luminometer (Turner Designs Model 20/20) according to the instructions for the Dual-luciferase assay system (Promega), and the relative light intensity was measured to reflect the luciferase activity. The firefly luciferase activity was normalized according to the Renilla luciferase activity and expressed as relative luciferase units (RLU) to reflect the promoter activity.

Immunoblotting analysis

Immunoblotting was performed as previously described (Qing *et al*. 2004). Cells were harvested in RIPA-DOC cell lysis buffer containing protease inhibitor 48 h after transfection. Cell lysates were separated on a 12% Tris-glycine or 16% Tris-tricine gels and immunoblotting was performed as described previously (Sun *et al*. 2006 *b*). A mouse anti-firefly luciferase monoclonal antibody (Anti-Luc) (Novus Biochemicals, USA) was used to detect firefly luciferase expression. Polyclonal C20 antibody against the last 20 amino acids of APP C-terminus was used to detect the full-length APP and c-terminal fragment (Sun *et al*. 2006*b*). Internal control β-actin was detected by monoclonal anti-β-actin antibody, AC15 (Sigma).

Quantitative RT–PCR

Total RNA was extracted with TRI reagent (Sigma) from the cells or the cortical tissue of the frontal cortex from human post-mortem brains. In order to completely remove probable residual DNA, DNase I (Invitrogen) was used to treat 2 μg of the RNA sample. Random hexamers and ThermoScript reverse transcriptase (Invitrogen) were used to generate the first-strand cDNA from 1 μg of total RNA sample.

Semi-quantitative PCR was performed in a 20-μl reaction mixture using Taq DNA polymerase and 0.25 μM of each primer. Twenty-six to 28 cycles were used to cover the linear region of the PCR amplification. A set of primers specific for human *BACE1* gene (5′-cggaattcgccaccatgctggtggatacaggcagc and 5′-cgggatcccacaatgctcttgtcatag) and primers specific for human glyceraldehyde-3 phosphate dehydrogenase (*hGAPDH*) gene (5′-tctggatcctcaccaccatggagaaggc and 5′-atactcgaggcagggatgatgttctg) were used to amplify 576-bp and 324-bp fragments, respectively. A set of primers specific for mouse *BACE1* gene (mBACE1-252, 5′-cccacagacgctcaacatcc and mBACE1-729r, 5′-cgagtggtcgatacctccaatg) and primers specific for mouse *β-actin* gene (mβActin-685f, 5′-actgccgcatcctcttcctc and mβActin-1087r, 5′-cgtactcctgcttgctgatcc) were used to amplify 477-bp and 402-bp fragments, respectively. The PCR products were loaded onto a 1.5% agarose gel and the result was analysed by Kodak Image Station 1000 software (PerkinElmer, USA). Quantitative real-time PCR was performed with SMART cycler II. Amplification reactions to detect relative cDNA levels were performed with the QuantiFast SYBR Green PCR kit and QuantiTect Primer Assay of human BACE1 and β-actin (Qiagen). The level of cDNA was calculated based on standard curve and data were normalized by the level of β-actin.

Gel shift assay (*GSA*)

NF-κB p65-enriched nuclear extract was prepared by transfecting NF-κB p65 expression cDNA plasmid (Tone *et al*. 2002) into HEK293 cells. GSA was performed as previously described (Christensen *et al*. 2004). Briefly, 70 fmol of 4NF-κB probe was labelled with [γ-^{32}P]ATP by T4 polynucleotide kinase and incubated with or without nuclear extract in gel shift binding buffer containing 4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris–HCl (pH 7.4), and 50 μg poly(dI-dC)·poly(dI-dC)/ml at room temperature for 20 min. For the gel supershifting assay, additional anti-NF-κB p65 antibody (Sigma, N8523) was added to the gel shift reaction mixture. The samples were analysed by 4% non-denaturing polyacrylamide gel electrophoresis (PAGE). The gel was subjected to autoradiography.

In the competition assays, 7 pmol (100 × excess) of NF-κB consensus oligonucleotide (5′-agttgagggactttcccaggc) (Promega) or unlabelled 4NF-κB probe served as competitors of the radiolabelled 4NF-κB probe. Moreover, four short double-stranded oligonucleotide probes (s-κB1, s-κB2, s-κB3 and s-κB4) corresponding to individual putative NF-κB-binding elements in the human *BACE1* promoter region were prepared by annealing the following four sets of primers: s-κB1f (5′-acacggtgaaaccccgtctc) and s-κB1r (5′-gagacggggtttcaccgtgt); s-κB2f (5′-aactggtgaaatcccatctc) and s-κB2r (5′-gagatgggatttcaccagtt); s-κB3f (5′-aaaacgaagattccctttca) and s-κB3r (5′-tgaaagggaatcttcgtttt); and s-κB4f (5′-attgtgtggaaccccactgc) and s-κB4r (5′-gcagtggggttccacacaat-3′), respectively. These four short competitor probes were used to determine which NF-κB-binding element in the human *BACE1* promoter region interacts with NF-κB p65.

Enzyme linked-immunosorbent assay (*ELISA*)

For the Aβ ELISA assay, culture medium was collected 48 h after transfection and treated with 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) (Sigma) to prevent protein degradation. The concentration of $A\beta_{40}$ was measured using Colorimetric ELISA kit for $A\beta_{1-40}$ (Biosource International Inc., USA) according to manufacturer's protocol. The concentration of the sample in pg/ml was calculated according to the standard absorbance–concentration curve.

For NF-κB p65 ELISA assay, the cortical tissues of the frontal cortex and cell pellets were homogenized in 1% SDS RIPA DOC buffer. After homogenization, cell lysates were sonicated and centrifuged for 10 min at 13 000 ***g*** at 4 °C. ELISA assay was performed using the PathScan Total NF-κB p65 Sandwich ELISA kit according to the manufacturer's instructions (Cell Signalling, USA). Briefly, 100 μl of each diluted cell lysate were added to the appropriate well (1.0 mg/ml), and the plate was incubated overnight at 4 °C. The contents of the wells were discarded. The wells were washed four times with 1x wash buffer, and incubated with 100 μl of detection antibody for 1 h at 37 °C. The wash procedure was repeated and the wells were incubated with 100 μl of HRP-linked secondary antibody for 30 min at 37 °C. After the wash procedure was repeated, samples were incubated with 100 μl TMB substrate for 10 min at 37 °C. The reaction was stopped with 100 μl of STOP solution. The absorbance at 450 nm was recorded.

Results

Increased NF-κB p65 and BACE1 level in the brain of AD patients

To investigate whether NF-κB signalling affects *BACE1* gene expression in AD pathogenesis, the

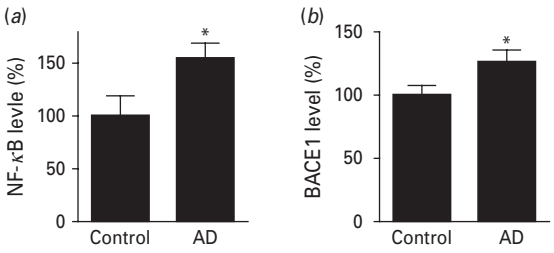

Fig. 1. Increased NF-κB p65 level in the brain of Alzheimer's disease (AD) patients. The cerebral cortical tissues of the frontal cortex from the post-mortem brains of 18 AD patients and 13 controls were used to extract protein and total RNA. The age range of patients at death was 65–79 yr. The region of the tissues was matched as closely as possible. (*a*) The concentration of RelA protein was determined by a colorimetric ELISA kit specific for human NF-κB p65. The values are expressed as means ± S.E.M. ($n = 39$ for AD, $n = 15$ for control). * $p < 0.05$ relative to controls by Student's t test. (*b*) Quantitative real-time PCR was used to determine the human *BACE1* mRNA level. The ratio of human *BACE1* to β-actin mRNA was expressed as means ± S.E.M. ($n = 39$ for AD, $n = 15$ for controls). * $p < 0.05$ relative to controls by Student's t test.

cerebral cortical tissues of the frontal cortex from the post-mortem brains of 18 AD patients and 13 controls were used for measuring protein level of NF-κB p65(RelA) and BACE1 expression. The age of AD patients was 74.89 ± 8.48 yr and for controls was 68.69 ± 10.09 yr ($p > 0.05$). There was no significant difference in the post-mortem intervals between AD (13.17 ± 7.97 h) and controls (18.46 ± 6.20 h) ($p > 0.05$). The results showed that the protein level of p65 significantly increased to 155.40 ± 13.39% in AD patient samples relative to control samples ($p < 0.05$) (Fig. 1*a*). Quantitative real-time RT–PCR was used to measure BACE1 mRNA in the brain samples. BACE1 mRNA levels were also markedly increased in the cortex of AD patients (126.40 ± 9.01% relative to controls, $p < 0.05$) (Fig. 1*b*). Our data show that both NF-κB p65 and BACE1 were elevated in the cortex of AD patients.

Identification of functional NF-κB-binding elements in the human **BACE1** *promoter region*

To determine the underlying mechanism by which NF-κB signalling modulates human BACE1 gene expression in AD pathogenesis, the promoter sequence of the human *BACE1* gene (GenBank AY162468) (Christensen *et al*. 2004) was analysed and four putative NF-κB-binding elements were identified based on the consensus binding sequence of NF-κB 5′-GGGRNNYYCC-3′ (Fig. 2*a*). To explore whether these putative NF-κB-binding elements physically interact with transcription factor NF-κB, GSA was performed (Fig. 2*b*). A double-stranded oligonucleotide probe (4NF-κB) containing all four putative NF-κB-binding elements of the *BACE1* promoter was synthesized and labelled with γ-^{32}P. The labelled ^{32}P-4NF-κB probe was incubated with nuclear extract obtained from NF-κB p65 transfected cells. A shifted protein–DNA complex band was detected after incubating the labelled ^{32}P-4NF-κB probe with nuclear extract (Fig. 2*b*, lane 2). Non-radioactive-labelled competitor NF-κB consensus oligonucleotide was also included in the experiment and the shifted band was completely abolished by addition of non-labelled NF-κB oligonucleotide (Fig. 2*b*, lane 3). A slower-migrating supershifted band was detected after anti-p65 antibody was incubated with the ^{32}P-labelled 4NF-κB probe and nuclear extract (Fig. 2*b*, lane 4). Furthermore, the shifted bands can be completely abolished by the non-labelled 4NF-κB oligonucleotide probe (Fig. 2*b*, lane 5). These results indicate that NF-κB p65 directly binds to the 4NF-κB oligonucleotides containing four putative NF-κB-binding elements from the human *BACE1* promoter region. To investigate whether the NF-κB-binding elements of *BACE1* gene promoter were functional, we inserted 4NF-κB double-stranded oligonucleotide in front of the firefly luciferase gene of pGL-pl (Cai *et al*. 2008) to generate p4NF-κB plasmid (Fig. 2*c*). NF-κB p65 knockout cells RelA-KO were transfected with pBACE1-4NF-κB, pGL-pl or pNF-κB-Luc plasmid with p65 expression plasmid pMTF-p65. Transfection with p65 expression plasmid resulted in robust p65 expression (Fig. 2*d*). p65 expression significantly increased luciferase activity in the cells transfected with pBACE1-4NF-κB by 10.25 ± 0.27-fold, compared to controls ($p < 0.001$) (Fig. 2*e*). p65 expression had no effect on luciferase activity in the cells transfected with vector control plasmid pGL-pl ($p > 0.05$) and markedly increased luciferase activity in the cells transfected with a positive control plasmid pNF-κB-Luc ($p < 0.001$) (Fig. 2*e*). Similar results were also observed by transfecting the plasmids into wild-type cells. p65 expression significantly increased luciferase activity in cells transfected with p4NF-κB and p65 plasmids by 13.93 ± 0.11-fold relative to controls (Fig. 2*f*). These results suggest that the 4NF-κB oligonucleotides containing four putative NF-κB-binding elements from the human *BACE1* promoter is able to respond to NF-κB signalling to modulate a downstream gene transcription.

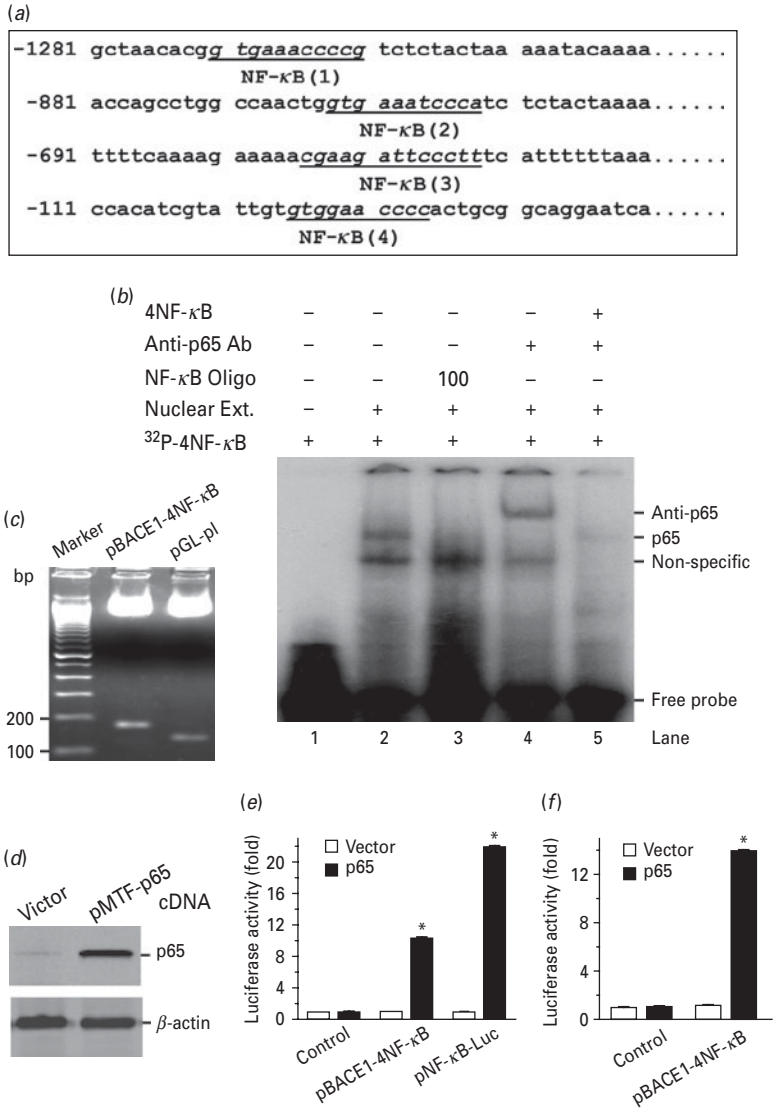

Fig. 2. Identification of NF-κB-binding elements in the human *BACE1* promoter region. (*a*) Sequence analysis reveals four putative NF-κB-binding sites within −1280 bp (relative to the transcription start site +1) of the human *BACE1* promoter region (GenBank AY162468) (Christensen *et al*. 2004). The nucleotide sequence of four putative NF-κB-binding elements, NF-κB (1), NF-κB (2), NF-κB (3) and NF-κB (4), are underlined. (*b*) Gel shift and gel supershift assays were performed as described in the Material and Methods section using a ^{32}P-labelled double-stranded oligonucleotides probe 4NF-κB, consisting of all four putative NF-κB-binding elements of the *BACE1* gene. Lane 1 is labelled probe alone without nuclear extract. Incubation of ^{32}P-4NF-κB with nuclear extracts retarded the migration rate of the labelled probe, which formed a new shifted DNA–p65 protein complex band (lane 2). Competition assays with 100× molar excess of unlabelled NF-κB consensus oligonucleotide (lane 3) or homologous 4NF-κB (lane 5) abolished the p65-shifting band. Anti-p65 antibody was used for super gel shift assay. The anti-p65 antibody supershifted the DNA nuclear protein p65 complex (lane 4). (*c*) pBACE1-4NF-κB plasmid contains the BACE1 4NF-κB oligonucleotide upstream of the firefly luciferase reporter gene of the pGL-pl plasmid. The plasmid construct was confirmed by sequencing and restriction enzyme digestion checking and the digested samples were analysed on a 1.5% agarose gel. (*d*) NF-κB p65 protein was robustly expressed by transecting cells with pMTF-p65 plasmid. p65 was detected by mouse monoclonal anti-p65 antibody. The enhancer activity of putative NF-κB-binding elements BACE1 4NF-κB was analysed by dual-luciferase reporter assays in (*e*) RelA-KO cells and (*f*) wild-type cells. Plasmid pBACE1-4NF-κB, pGL-pl or pNF-κB-Luc was co-transfected with NF-κB p65 expression plasmid or empty vector pMTF. Transfection efficiency was normalized by Renilla luciferase expression of pCMV-RLuc. The values are expressed as means ± S.E.M. ($n=3$). * $p<0.001$ relative to controls by ANOVA with *post-hoc* Newmann–Keuls test.

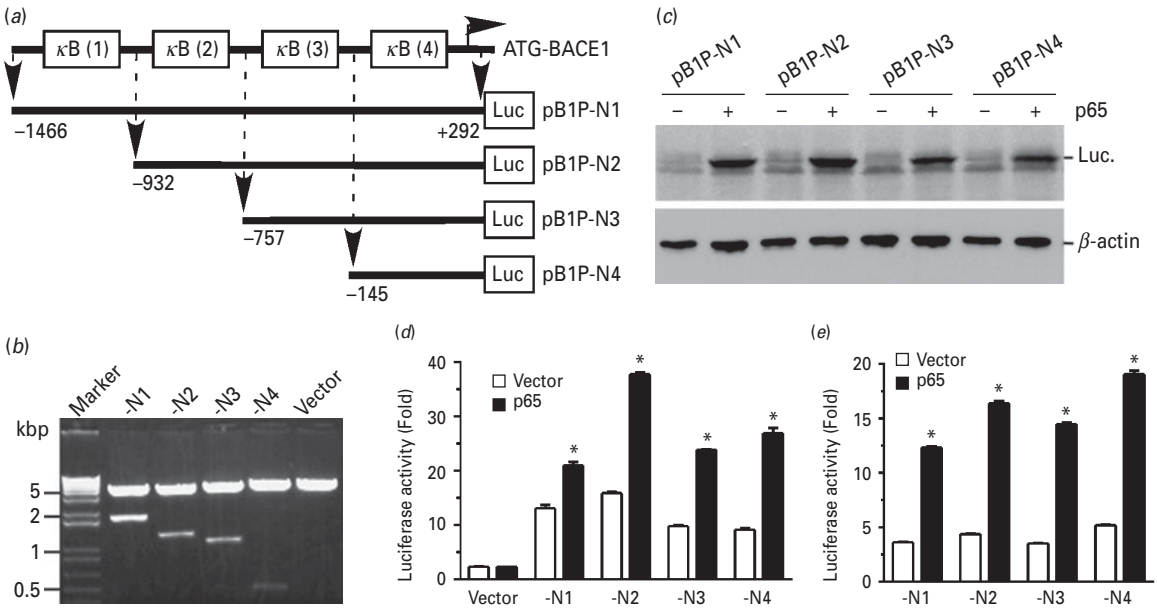

Fig. 3. *BACE1* promoter activity is up-regulated by NF-κB p65. (*a*) Schematic diagram of deletion plasmids containing different human *BACE1* promoter fragments in front of the firefly luciferase reporter gene of the promoter-less pGL3-basic plasmid. (*b*) The deletion plasmids corresponding to (*a*) were confirmed by sequencing and restriction enzyme digestion checking and the digested samples were analysed on a 1% agarose gel. Vector size is 4.7 kb and the *BACE1* promoter fragment insert sizes range from 0.43 to 1.75 kbp. (*c*) HEK293 cells were co-transfected with *BACE1* reporter plasmids and NF-κB p65 expression plasmid or empty vector pMTF. Cell lysates were collected 48 h after transfection and subjected to Western blot analysis on a 12% Tris-glycine SDS–PAGE using monoclonal anti-luciferase antibody to detect the level of luciferase protein and AC15 antibody to detect β-actin protein levels. *BACE1* promoter deletion plasmids and NF-κB p65 expression plasmid or control vectors were co-transfected into (*d*) HEK293 cells and (*e*) N2a cells. The transfected cells were harvested with passive lysis buffer and luciferase activity was measured. Renilla luciferase activity was used to normalize transfection efficiency. The values are expressed as means ± S.E.M. ($n=3$). * $p<0.001$ relative to controls by ANOVA with *post-hoc* Newmann–Keuls test.

NF-κB p65 regulates human BACE1 *promoter activity*

In order to determine the effect of NF-κB p65 on human *BACE1* gene expression, we generated four *BACE1* promoter plasmids, a series of human *BACE1* promoter deletion constructs pB1P-N1, -N2, -N3, and -N4 with sequential eliminations of one upstream putative NF-κB-binding element were also cloned into promoter-less pGL3-basic plasmid (Fig. 3*a*, *b*). The *BACE1* reporter plasmids were co-transfected into cells with either NF-κB p65 expression plasmid or empty vector pMTF. Luciferase protein expression level was detected by anti-luciferase antibody and normalized by internal β-actin protein levels. Western blot analysis revealed a significant increase in luciferase expression by NF-κB p65 expression in cell lysates from cells co-transfected with all *BACE1* reporter plasmids and plasmid (Fig. 3*c*). To confirm our Western blot results, we used a dual-luciferase reporter assay to examine luciferase expression of the *BACE1* reporter plasmids in HEK293 cells co-transfected with NF-κB p65 expression plasmid or empty vector pMTF. Firefly luciferase activity was normalized by internal Renilla luciferase expression. Consistent with our Western blot results, a significant elevation of promoter activity was detected in cells co-expressing *BACE1* reporter plasmid and NF-κB p65 (Fig. 3*d*). Compared to empty vector controls, co-transfection of NF-κB p65 expression plasmid resulted in increased luciferase activity in the cells transfected with pB1P-N1, pB1P-N2, pB1P-N3 and pB1P-N4 by 1.61-fold (13.09 ± 0.04 to 21.02 ± 0.07, $p<0.001$), 2.38-fold (15.88 ± 0.20 to 37.75 ± 0.35, $p<0.001$), 2.43-fold (9.81 ± 0.15 to 23.87 ± 0.02, $p<0.001$) and 2.94-fold (9.15 ± 0.28 to 26.90 ± 0.99, $p<0.001$), respectively. No significant increase in luciferase activity was detected in cells co-transfected with control vector and NF-κB p65 plasmids. Similar results were also obtained by performing the experiments in N2a neuronal cells

(Fig. 3*e*). Taken together, these results demonstrate that NF-κB p65 up-regulates human *BACE1* promoter activity.

BACE1 *promoter activity is regulated through two distinct NF-κB-binding elements*

To investigate which one of the putative NF-κB-binding elements of *BACE1* interacted with NF-κB p65, we synthesized four short probes (s-κB1, s-κB2, s-κB3 and s-κB4) as competitors of the 4NF-κB probe in GSA. Each short probe covered the sequence of an individual putative NF-κB-binding element identified in the *BACE1* promoter region. Complex formation between nuclear extract and labelled 4NF-κB probe was abolished when either 4NF-κB, s-κB1 or s-κB4 (Fig. 4*a*; lanes 3, 4, 7), but not s-κB2 or s-κB3 (lanes 5 and 6), was applied. The data suggests that the first and the fourth NF-κB-binding elements in *BACE1* gene are able to interact with NF-κB p65. To determine whether these two *cis*-acting binding elements mediate the regulatory effect of NF-κB signalling on human *BACE1* gene expression, we generated *BACE1* reporter plasmids containing a mutated NF-κB-binding element by site-directed mutagenesis (Fig. 4*b*). Mutations in the first or fourth NF-κB-binding element significantly reduced luciferase activity in cells transfected with pB1P-κ1 m or pB1P-κ4 m by $56.42 \pm 0.78\%$ and 55.65 ± 0.66, relative to the wild-type plasmid pB1P-N1 ($p < 0.001$) (Fig. 4*c*). Consistent with the luciferase activity data, when NF-κB p65 expression plasmid was co-transfected with the wild-type plasmid pB1P-N1, luciferase protein was robustly expressed (Fig. 4*d*). However, mutations in the first or the fourth NF-κB-binding element inhibited the luciferase protein expression induced by p65 expression, resulting in reduction in luciferase protein level by $83.94 \pm 0.72\%$ and $85.36 \pm 0.13\%$ ($p < 0.05$ relative to wild-type plasmid pB1P-N1) (Fig. 4*e*). These results demonstrate that mutating the NF-κB p65-binding elements in the *BACE1* promoter region inhibits the ability of NF-κB p65 to increase luciferase expression. Similar results were also observed in the neuronal cell line N2a and SH-SY5Y cells (data not shown). Therefore, these two NF-κB-binding elements of the human *BACE1* promoter are important for regulation of *BACE1* promoter activity by NF-κB signalling.

p65 up-regulates human BACE1 *gene transcription and facilitates β-secretase processing of APP to generate Aβ production*

To investigate whether NF-κB regulates human *BACE1* gene expression at the transcriptional level, semi-quantitative RT–PCR was performed to measure endogenous BACE1 mRNA levels in cells transfected with empty vector or NF-κB p65 expression plasmid (Fig. 5*a*). When wild-type cells were transfected with NF-κB p65 expression plasmid, the mRNA level of *BACE1* was elevated by $476.6 \pm 21.68\%$ ($p < 0.0001$ relative to controls) (Fig. 5*b*). Disruption of p65 gene in the RelA-KO cells inhibited *BACE1* gene transcription, resulting in a marked decrease in endogenous *BACE1* mRNA level to $37.15 \pm 1.69\%$ ($p < 0.0001$) (Fig. 5*c*, *d*). Restoring p65 expression by transfecting p65 expression plasmid into the RelA-KO cells significantly enhanced the endogenous *BACE1* gene transcription, and the *BACE1* mRNA level was increased by $180.90 \pm 9.61\%$ in p65-transfectd RelA-KO cells compared to vector control-transfected RelA-KO cells ($p < 0.005$) (Fig. 5*e*, *f*). Herein, we demonstrate that NF-κB p65 regulates human *BACE1* expression at the transcriptional level, resulting in elevation in *BACE1* mRNA synthesis. To examine the BACE1 protein level, 20E2 cells, a cell line stably expressing Swedish mutant APP695 in HEK293 cells (Qing *et al*. 2004), were transfected with control vector pMTF or NF-κB p65 expression plasmid. Our result showed that p65 overexpression significantly increased BACE1 protein level by $232.54 \pm 12.86\%$ ($p < 0.01$ relative to controls) (Fig. 5*g*). To examine whether APP processing is also modulated by NF-κB signalling through its effect on *BACE1* gene expression, the level of APP and its C-terminal fragments as well as Aβ production were measured. p65 expression significantly increased the amount of C99 in 20E2 cells by $152.29 \pm 6.03\%$ in the presence of NF-κB p65 relative to controls ($p < 0.001$) (Fig. 5*h*, *i*). There was no significant difference in APP level between p65 transfected cells and controls ($p > 0.05$). The data indicates that p65 expression up-regulated APP processing at the β-secretase cleavage site by elevating *BACE1* gene expression, resulting in increased C99 production. To investigate the effect of NF-κB p65 on Aβ generation, we measured Aβ levels by colorimetric ELISA. The results showed that NF-κB p65 expression significantly increased total Aβ protein concentration by $134.90 \pm 5.74\%$ in SAS1 cells, a stable SH-SY5Y cell stably expressing Swedish mutant APP695 (Sun *et al*. 2006*a*) ($p < 0.0001$) (Fig. 5*j*). IκBα expression plasmid was also transfected into SAS1 cells and IκBα transfection reduced Aβ protein levels to $92.24 \pm 2.68\%$ ($p < 0.05$). Hence, Aβ production was altered by NF-κB p65 in neuronal cell lines.

NSAIDs inhibit TNF-α-induced BACE1 *transcription*

Inflammation is one of major pathological changes in AD brains and NF-κB signalling plays an

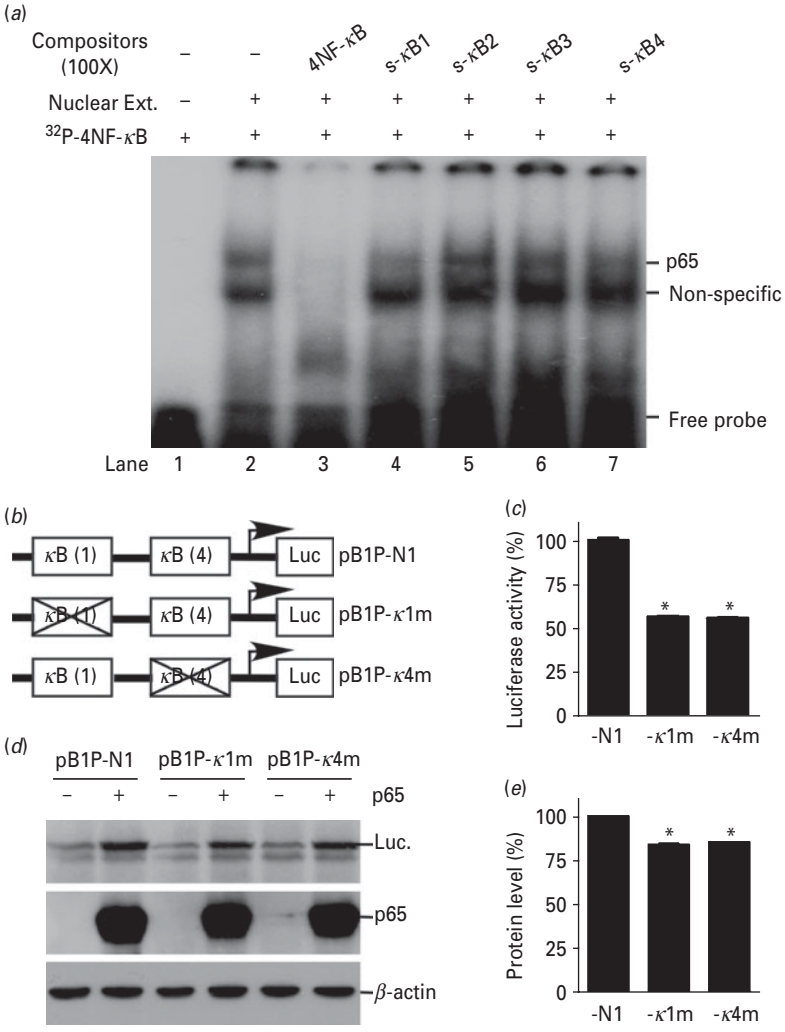

Fig. 4. Two distinct NF-κB-binding elements mediate the regulation of *BACE1* promoter activity by NF-κB signalling. (*a*) Specific NF-κB p65-binding elements in the human *BACE1* promoter region were determined by gel shift assay. In the competition assays, the addition of excess unlabelled homologous 4NF-κB probe abolished the complex formations (lane 3). The specific p65-shifted band was abolished by addition of excess s-κB1 or s-κB4 probes (lanes 4 and 7), but not addition of excess s-κB2 or s-κB3 probes (lanes 5 and 6). (*b*) Illustration of the human *BACE1* promoter reporter plasmids where mutated NF-κB-binding elements are marked with an 'X'. The mutations were confirmed by sequencing. (*c*) The wild-type (pB1P-N1) or NF-κB-binding-site mutant *BACE1* reporter plasmids (pB1P-κ1m or pB1P-κ4 m) were co-transfected into HEK293 cells with NF-κB p65 expression plasmid or empty vector pMTF. Luciferase activity was measured after 48 h. Renilla luciferase activity was used to normalize transfection efficiency. Luciferase activity from cells transfected with pB1P-N1 and NF-κB p65 expression plasmid served as wild-type control. The first and the fourth NF-κB-binding-site mutation significantly reduced BACE1 promoter activity ($n=3$). * $p<0.01$ relative to controls by Student's t test. (*d*) Western blot analysis was used to determine the level of luciferase protein generated in HEK293 cells after co-transfection of *BACE1* reporter plasmids with or without NF-κB p65 expression plasmid. Cell lysates were collected 48 h after transfection and electrophoresed by 12% Tris-glycine SDS–PAGE. Monoclonal anti-NF-κB p65 antibody was also used to detect the level of NF-κB p65. A robust protein level of NF-κB p65 was detected in cells expressing NF-κB p65. AC15 was included to detect β-actin protein levels. Comparing to the control plasmid pB1P-N1, mutation of the NF-κB-binding elements leads to inhibition of luciferase protein expression. (*e*) Quantitative analysis of luciferase expression level. Values are means ± S.E.M. ($n=3$). * $p<0.05$ relative to controls by Student's t test.

important role in inflammation and oxidative stress (Tong *et al*. 2005). TNF-α is a strong activator of NF-κB signalling pathways. To investigate whether NSAIDs could reduce the *BACE1* up-regulation induced by NF-κB signalling activation, HEK293 cells were transfected with the human BACE1 promoter constructs

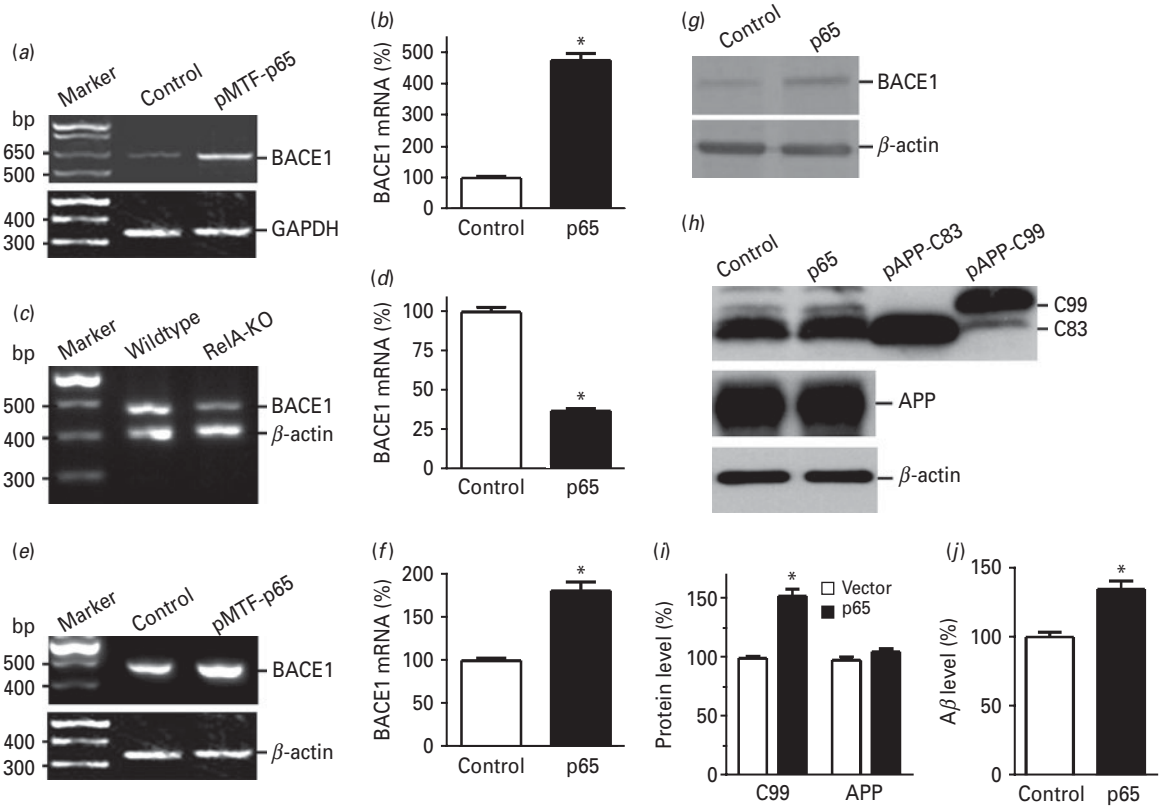

Fig. 5. NF-κB p65 up-regulates human *BACE1* gene expression at the transcriptional level and potentiates C99 and Aβ generation. RNA was extracted from cells. Semi-quantitative RT–PCR was performed to measure endogenous human *BACE1* mRNA levels with specific primers recognizing coding sequences of the *BACE1* gene and GAPDH or β-actin level as an internal control. PCR products after 26 cycles were analysed on 1% agarose gel. (*a*) HEK293 cells were transfected with empty vector as control or p65 expression vector pMTF-p65 and the ratio of human BACE1 to human GAPDH mRNA was analysed (*b*). Endogenous *BACE1* mRNA was significantly increased in cells transfected with NF-κB p65 expression plasmid. (*c*, *d*) The *BACE1* mRNA levels in wild-type and RelA-KO were analysed. Disruption of p65 in RelA-KO cells markedly reduced endogenous *BACE1* mRNA levels. (*e*, *f*) RelA-KO cells were transfected with empty vector or pMTF-p65, and transfection of p65 into RelA-KO cells significantly increased *BACE1* mRNA levels. The values are shown as means ± S.E.M. ($n=3$). * $p<0.001$ relative to controls by Student's *t* test. 20E2 cells were transfected with empty vector pMTF or NF-κB p65 expression plasmid, and Western blot analysis was performed to detect protein levels of (*g*) *BACE1*, (*h*) full-length APP, C83 and C99 on a 16% Tris-tricine SDS–PAGE. β-actin was detected by monoclonal anti-β-actin antibody AC-15. Plasmid pAPP-C99 and pAPP-C83 were transfected into cells and the expressed C99 and C83 proteins were used as the marker. (*i*) Quantitative analysis of C99 and full-length APP protein levels. The values are expressed as means ± S.E.M. ($n=3$). * $p<0.005$ relative to controls by the Student's *t* test. (*j*) SAS cells were transfected with empty vector or NF-κB p65 expression plasmid and the culture supernatant was collected. The concentration of total Aβ was determined by colorimetric ELISA kit. The values are expressed as means ± S.E.M. ($n=12$). * $p<0.001$ relative to controlsby Student's *t* test.

and treated with TNF-α for transcriptional activation. NSAIDs were then added to the cell cultures. Compared to controls, TNF-α treatment resulted in significant increase in luciferase activity in the cells transfected with the human BACE1 promoter pB1P-N1 plasmid ($p<0.005$) and addition of aspirin inhibited the *BACE1* transcriptional activation induced by TNF-α ($p<0.005$ relative to TNF-α and $p>0.05$ relative to controls) (Fig. 6*a*). To further confirm the effect of NSAIDs on NF-κB-mediated transcriptional activation, the cells were transfected with BACE1-NF-κB promoter construct pBACE1-4NF-κB. Consistent with pB1P-N1 plasmid results, TNF-α significantly induced BACE1-NF-κB promoter activation ($p<0.005$) and the NSAIDs aspirin, ibuprofen and indomethacin significantly blocked the TNF-α-induced BACE1-NF-κB promoter activation ($p<0.005$) (Fig. 6*b*). Our data clearly demonstrate that NSAIDs inhibit

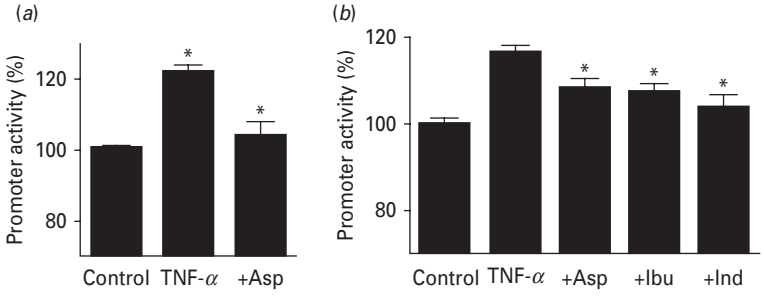

Fig. 6. NSAIDs inhibit TNF-α-induced *BACE1* transcription. (*a*) HEK293 cells were co-transfected with *BACE1* reporter plasmid pBACE1-N1 and pCMV-RLuc. Transfected cells were treated with vehicle solution (control), 10 ng/ml TNF-α alone (TNF-α) or with 2.5 mM aspirin (+Asp) for 24 h. The transfected cells were harvested with passive lysis buffer and luciferase activity was measured. Renilla luciferase activity was used to normalize for transfection efficiency. The values are expressed as means ± S.E.M. ($n=3$). * $p<0.005$ by ANOVA with *post-hoc* Newmann–Keuls test. (*b*) HEK293 cells were co-transfected with *BACE1* reporter plasmid pBACE1-NF-κB-Luc and pCMV-RLuc. Transfected cells were treated with vehicle solution (control), 10 ng/ml TNF-α alone (TNF-α), with 2.5 mM aspirin (+Asp), 250 μM (S)-ibuprofen (+Ibu) or 50 μM indomethacin (+Ind). The transfected cells were harvested with passive lysis buffer and luciferase activity was measured. The values are expressed as means ± S.E.M. ($n=3$). * $p<0.005$ by ANOVA with *post-hoc* Newmann–Keuls test.

TNF-α-induced *BACE1* transcription via its NF-κB *cis*-acting elements.

Discussion

AD, the most common neurodegenerative disease leading to dementia, is pathologically characterized by Aβ deposition in the brain. Aβ is generated by sequential cleavages of APP by β- and γ-secretases. BACE1 cleavage of APP at β-secretase site is essential for Aβ generation. Lower *BACE1* transcription and tightly controlled *BACE1* gene translational initiation result in the minority of APPs undergoing amyloidogenic process and relatively lower Aβ production under normal conditions, and a slight increase of *BACE1* induces a marked elevation in Aβ production and facilitate neuritic plaque formation in the pathological condition (Li *et al*. 2006; Zhou & Song, 2006). In our previous studies, we have characterized the human *BACE1* promoter region and demonstrated that *BACE1* gene expression is tightly controlled at transcription and translation level (Christensen *et al*. 2004; Zhou & Song, 2006). Abnormal *BACE1* gene expression and elevated BACE1 activity has been implicated in AD pathogenesis (Sun *et al*. 2006*a*, *c*). It was reported that a rat BACE1 promoter contains a NF-κB-binding element (Bourne *et al*. 2007) and exacerbated Aβ levels modulate rat BACE1 promoter activity via NF-κB-dependent pathways (Buggia-Prevot *et al*. 2008). NF-κB activity can be induced by various stimuli, such as TNF, IL-1 and glutamate and, particularly, Aβ and secreted forms of APP were reported to activate NF-κB in neuronal and glial cells in AD (Barger *et al*. 1995; Behl *et al*. 1994; Guo *et al*. 1998). Increased NF-κB activity has been detected in neurons and astrocytes in the vicinity of Aβ plaques in AD brains, and compared to the particulate fractions of temporal cortex from control brains, those from AD brains showed augmented NF-κB p65 protein levels (Kitamura *et al*. 1997; Terai *et al*. 1996). In this study, we show, for the first time, that NF-κB physically interacts with the human *BACE1* promoter region and regulates human *BACE1* gene expression at the transcription level.

AD is a chronic neurodegenerative disease and a low level of oxidative stress and exacerbated inflammatory responses induced by Aβ may depend on transcription factors to exert neurotoxic effects, and the regulation of NF-κB-dependent gene transcription may mediate the neurodegenerative effect attributed by Aβ toxicity in AD (Behl *et al*. 1994; Yankner *et al*. 1990). Anti-apoptotic Bcl-2 overexpression is suggested to prevent Aβ-induced cell death in neurons, and this protective effect is correlated with the inhibition of Aβ-stimulated NF-κB activation (Christensen *et al*. 2004; Saille *et al*. 1999; Vinet *et al*. 2002). However, the further effect of NF-κB signalling activation due to Aβ-induced oxidative stress and exacerbated inflammatory responses on APP processing is not clear. We found that there are four putative NF-κB-binding elements in the human *BACE1* promoter, two of which interact with NF-κB p65. Our study shows that NF-κB p65 significantly up-regulates *BACE1* promoter

activity through physical interaction with NF-κB-binding elements on the *BACE1* promoter, and mutations in the *cis*-acting elements disrupting p65 binding diminish the regulatory effects of NF-κB on *BACE1* promoter activity. Our study also shows that NF-κB p65 expression increased endogenous *BACE1* transcription, and targeted disruption of NF-κB p65 gene in RelA knockout cells reduced *BACE1* gene expression. NF-κB p65 not only elevates *BACE1* gene expression, but also increases C99 and Aβ generation. Furthermore, we found that both BACE1 and p65 are significantly increased in the brains of AD patients. Taken together, our results clearly demonstrate that NF-κB-binding elements on the *BACE1* promoter are able to regulate *BACE1* gene transcription, and activation of the NF-κB signalling pathway can facilitate *BACE1* gene expression and APP processing to generate more Aβ. This could create a vicious circle exacerbating oxidative stress and inflamatory responses induced by abnormal Aβ aggregation and plaque formation, resulting in activation of NF-κB signalling, in turn triggering more Aβ production by up-regulating *BACE1* gene expression. Our study suggests that increased *BACE1* expression mediated by NF-κB signalling in the brain may be one of the novel molecular mechanisms underlying the development of AD pathogenesis in some sporadic cases. Future studies are warranted to further investigate the signals which are responsible for increased NF-κB activity in AD.

Some NSAIDs have also been demonstrated to have therapeutic potential in AD treatment by altering amyloidogenic APP processing and reducing Aβ generation (Blasko *et al*. 2001; Sastre *et al*. 2006; Weggen *et al*. 2001). We have previously demonstrated that H_2O_2 is able to up-regulate *BACE1* gene transcription and increase Aβ generation (Tong *et al*. 2005). NF-κB p65 expression in the present study significantly increases *BACE1* mRNA and facilitates C99 and Aβ production. Furthermore, our data show that NSAIDs inhibit *BACE1* transcriptional activation induced by strong NF-κB activator TNF-α. Brain inflammation is one of the aggregating factors contributing to neurodegeneration in AD and could potentiate *BACE1* expression, leading to further overproduction of Aβ through the NF-κB signalling pathway. Our results suggest that NSAIDs can block the inflammation-induced *BACE1* transcription and Aβ production. Due to the vital role of *BACE1* in Aβ generation in the pathological progression of AD (Li *et al*. 2006), our study suggests that inhibition of NF-κB-mediated *BACE1* expression may be a valuable drug target for AD therapy.

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Statement of Interest

None.

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