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AP-2 β REGULATES AMYLOID BETA-PROTEIN STIMULATION OF APOLIPOPROTEIN E TRANSCRIPTION IN ASTROCYTES

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Abstract

Two key players involved in Alzheimer's disease (AD) are amyloid beta protein (A β) and apolipoprotein E (apoE). A β increases apoE protein levels in astrocytes which is associated with cholesterol trafficking, neuroinflammatory responses and A β clearance. The mechanism for the increase in apoE protein abundance is not understood. Based on different lines of evidence, we propose that the beta-adrenergic receptor (β AR), cAMP and the transcription factor activator protein-2 (AP-2) are contributors to the A β -induced increase in apoE abundance. This hypothesis was tested in mouse primary astrocytes and in cells transfected with an apoE promoter fragment with binding sites for AP-2. A β (42) induced a time-dependent increase in apoE mRNA and protein levels which were significantly inhibited by β AR antagonists. A novel finding was that A β incubation significantly reduced AP-2 α levels and significantly increased AP-2 β levels in the nuclear fraction. The impact of A β -induced translocation of AP-2 into the nucleus was demonstrated in cells expressing AP-2 and incubated with A β (42). AP-2 expressing cells had enhanced activation of the *apoE* promoter region containing AP-2 binding sites in contrast to AP-2 deficient cells. The transcriptional upregulation of apoE expression by A β (42) may be a neuroprotective response to A β -induced cytotoxicity, consistent with apoE's role in cytoprotection.

Keywords

Alzheimer's disease; amyloid beta-protein; apolipoprotein E; activator protein-2; beta-adrenergic receptor; neuroprotection

1. Introduction

ApoE is involved in neurodegeneration and regeneration and its gene expression and protein levels are elevated after neuronal injury and in Alzheimer's disease (AD) (Cedazo-Mínguez,

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2007; Haasdijk et al., 2002; Ignatius et al., 1986; Li et al., 2010a; Seitz et al., 2003; Yamada et al., 1995; Zarow and Victoroff, 1998). Metabolism and clearance of the amyloid beta protein (A β) is associated with apoE (Fan et al., 2009; Deane et al., 2008). A β increases cellular apoE levels, but the mechanism for this increase has not been established (Hu et al., 1998; Igbavboa et al., 2006; Igbavboa et al., 2003; LaDu et al., 2000; LaDu et al., 2001). Different lines of evidence have lead us to propose that A β may be stimulating apoE transcription involving the beta-adrenergic receptor (β AR), cAMP, and the transcription factor, activator protein-2 (AP-2). Brain tissue of patients with AD showed increased apoE mRNA levels as compared with control individuals (Yamada et al., 1995; Yamagata et al., 2001; Zarow et al., 1998) although there have been reports that apoE mRNA levels were lower in AD patients or unchanged (Oyama et al., 1995; Poirier et al., 1991). Cyclic AMP (cAMP), a second messenger that is also upregulated in AD and after astrocyte activation, increased apoE expression and protein secretion (Cedazo-Mínguez et al., 2001; LaDu et al., 2001; Martinez et al., 2001; Prapong et al., 2001). A β directly binds to the β AR and alters its internalization and degradation (Wang et al., 2010; Wang et al., 2011). A β (42)-induced stimulation of apoE protein levels in mouse primary astrocytes by activation of β AR and a cAMP-dependent pathway (Igbavboa et al., 2006). The transcription factor AP-2 regulates apoE gene expression in astrocytoma cells which is mediated by cAMP (García et al., 1996). AP-2 is an inducible cell type-specific transcription factor family consisting of five closely related proteins (α , β , γ , δ and ϵ) that regulate the expression of specific target genes (Damberg, 2005; Eckert et al., 2005). AP-2 α and β are the most abundant isoforms in the brain. The proximal region of the *apoE* has AP-2 consensus sequences (Du et al., 2005; Lahiri, 2004; Maloney et al., 2007).

The present study determined whether the A β -induced increase of apoE protein abundance in astrocytes is due to stimulation of apoE gene expression mediated by binding of the transcription factor AP-2 to the *apoE* promoter region. We tested this hypothesis in mouse primary astrocytes and in cells transfected with a luciferase reporter gene under the control of an *apoE* promoter fragment containing AP-2 binding sites. Based on our previous studies, we used soluble untreated A β (42) because this form, but not aggregated or oligomeric A β , increased apoE protein levels and altered cholesterol distribution in the Golgi complex and plasma membrane of mouse primary astrocytes (Igbavboa et al., 2006; Igbavboa et al., 2003; Igbavboa, et al., 2009). In the present study, we demonstrate that A β (42) increased apoE mRNA levels which were inhibited by β AR antagonists. A β (42) significantly increased AP-2 β levels but significantly reduced AP-2 α levels in the nuclear extract. Cells expressing AP-2 showed A β (42)-induced activation of a co-expressed luciferase reporter gene construct under the control of an *apoE* promoter fragment containing AP-2 binding sites in contrast to cells not expressing AP-2. These novel findings demonstrate for the first time that A β (42) stimulates apoE gene expression by specifically inducing activation of the transcription factor AP-2 β .

2. RESULTS

2.1 A β (42) increases apoE mRNA expression levels which is inhibited by β AR antagonists in mouse primary astrocytes

Data in Figure 1A show that A β (42) increased apoE mRNA levels in a time-dependent manner. Significant stimulation occurred after the 30 min A β treatment with the peak level reached after 60 min. Data were normalized to β -actin mRNA whose expression level was unaffected by A β (42). We also found that A β (42) treatment significantly increased apoE protein levels (Fig. 1B) which is in agreement with earlier reports (Igbavboa et al., 2006; Igbavboa et al., 2003). This increase in apoE protein levels is consistent with A β increasing apoE mRNA levels seen in Figure 1A.

It has been reported that A β (42) stimulation of apoE protein abundance was inhibited by β AR antagonists (Igbavboa et al., 2006). Therefore, it was determined whether the stimulatory effect of A β (42) on apoE mRNA seen in Figure 1A could be inhibited by β AR antagonists. The non-selective antagonist propranolol and the selective beta-adrenergic receptor antagonists, betaxolol for the β_1 receptor and ICI 118551 for the β_2 receptor were used. Incubation conditions and drug concentrations were the same as previously reported for effects of A β (42) on apoE protein levels ((Igbavboa et al., 2006). Data in Figure 1A showed that A β (42) significantly increased apoE mRNA levels as determined by endpoint RT-PCR. Those results were confirmed as seen in Figure 1C using qRT-PCR. Moreover, the A β -induced stimulation of apoE mRNA levels was significantly inhibited by the non-selective antagonist propranolol ($p \leq 0.01$) and the β_2 AR antagonist ICI 118551 ($p \leq 0.005$), with ICI having a greater inhibitory effect as shown in Figure 1C. The β_1 AR antagonist betaxolol did not significantly inhibit effects of A β (42) on apoE mRNA expression levels (Fig. 1C). This absence of a significant effect of the β_1 AR antagonist on apoE mRNA levels is similar to what we observed on apoE protein levels ((Igbavboa et al., 2006). We now demonstrate in mouse primary astrocytes that both apoE mRNA and protein levels are increased by A β (42) and that these stimulatory effects of A β (42) are inhibited by β_2 AR antagonists.

2.2 A β (42) effects on AP-2 α and β protein levels in the nuclear fraction and cell lysate in mouse primary astrocytes

We propose that A β (42) increases apoE protein abundance through a pathway that involves β AR activation and binding of the transcription factor AP-2 to the apoE promoter region. Reports indicate that of the five AP-2 isoforms, AP-2 α and AP-2 β are the most abundant isoforms in the brain; AP-2 γ is co-expressed with AP-2 α and AP-2 β in several brain regions, but its expression level is the lowest among the three (Coelho et al., 2005; Damberg, 2005; Moser et al., 1995; Oulad-Abdelghani et al., 1996; Shimada et al., 1999). Effects of A β (42) on protein levels of AP-2 α and AP-2 β were determined in the nuclear extract of primary astrocytes. A β incubation significantly reduced AP-2 α levels (Fig. 2A) and significantly increased AP-2 β levels (Fig. 2B) in the nuclear extract. Total protein levels of AP-2 α and AP-2 β in the cell lysates of A β treated and control cells did not differ (Fig. 2C and 2D) suggesting that the A β -induced increase of AP-2 β and the reduction of AP-2 α in the nuclear fraction were due to a redistribution of the proteins and not synthesis or inhibition of degradation.

2.3. A β (42) stimulates apoE promoter activity

Data in Figure 2B showed that A β (42) significantly increased AP-2 β protein levels in the nuclear fraction of astrocytes. There is evidence that the proximal *apoE* promoter activity in astrocytes is regulated by binding of the transcription factor AP-2 to two sites located on the *apoE* proximal promoter region (García et al., 1996). We determined whether A β (42) would activate the *apoE* promoter region containing AP-2 binding sites. DITNC1 (immortalized rat astrocytes) and AP-2 deficient HepG2 cells were transfected with a luciferase reporter gene under the control of an *apoE* promoter fragment that included the AP-2 binding sites. Cells were incubated with A β (42). Figure 3 clearly shows that luciferase activity was significantly increased by A β (42) in DITNC1 cells indicative of AP-2 binding to the *apoE* promoter. HepG2 cells that are AP-2 deficient, did not display changes in luciferase activity when treated with A β (42) (Fig. 3).

3. Discussion

Increased apoE mRNA levels have been observed after CNS injury as well as in brains of AD mouse models and AD patients (Cedazo-Mínguez et al., 2001; Haasdijk et al., 2002;

Ignatius et al., 1986; LaDu et al., 2001; Seitz et al., 2003; Yamada et al., 1995; Zarow et al., 1998). A β metabolism and clearance is associated with apoE (Fan et al., 2009; Deane et al., 2008). ApoE upregulation may act to reduce astrocyte activation and the prevention and repair of neuronal damage caused by A β (Lynch et al., 2001). It is well documented that apoE-dependent transport of cholesterol from astrocytes to neurons is important in maintaining optimal neuronal function (Göritz et al., 2002; Michikawa et al., 2001; Vance et al., 2005). Our group has described how A β (42) affects cholesterol transport in the Golgi complex and plasma membrane of astrocytes and revealed that these effects were associated with apoE and caveolin-1 (Igbavboa et al., 2006; Igbavboa et al., 2003; Igbavboa et al., 2009). Effects of A β on apoE, and cholesterol distribution could impact lipid trafficking within the cell and between astrocytes and neurons. A β increases apoE protein levels but the mechanism is not clearly understood (Igbavboa et al., 2006; Igbavboa et al., 2003; Kimura et al., 2004; LaDu et al., 2000; LaDu et al., 2001). Different lines of evidence (García et al., 1996; Igbavboa et al., 2006) provided the framework for us to propose that the A β -induced increase in apoE protein abundance may involve the β AR, cAMP and the transcription factor AP-2. We previously reported that A β (42) increased apoE protein and cAMP levels which could be inhibited by β AR antagonists in astrocytes (Igbavboa et al., 2006). Recent data showed that A β directly binds to the β_2 AR (Wang et al., 2010). An earlier study found that *apoE* promoter activity in astrocytes was upregulated by cAMP-dependent activation of the transcription factor AP-2, for which there are two binding sites located in the *apoE* promoter's proximal region (García et al., 1996). Therefore, the focus of the present study was to test the hypothesis that one mechanism for the A β -induced increase in apoE protein expression is the transcriptional upregulation of *apoE* due to β AR-mediated activation of the transcription factor AP-2. Our results show that apoE mRNA and protein levels were increased after A β (42) treatment in astrocytes and these effects were mediated in part by activation of the β AR. A β effects on apoE mRNA expression in the present study were inhibited by β AR antagonists, particularly ICI 118551 which is selective for the β_2 receptor subtype. A β effects on the β_2 receptor subtype are consistent with the recent finding that A β directly binds to the β_2 AR and stimulates its internalization and degradation (Wang et al., 2010; Wang et al., 2011). The β_1 AR antagonist betaxolol in the present study did not significantly inhibit the stimulatory effect of A β (42) on apoE mRNA expression.

ApoE gene expression is regulated by AP-2 (García et al., 1999; García et al., 1996). We discovered that out of the five characterized AP-2 isoforms, α and β were abundantly expressed in mouse primary astrocytes. When cells were challenged with A β (42), AP-2 α showed a modest but significant decrease in the nucleus while AP-2 β showed a significant increase. Total protein levels of both AP-2 isoforms were not changed after A β (42) treatment, indicating that A β was affecting nuclear distribution of these AP-2 isoforms. The mechanism for this regulation is unclear as is the functional consequences of reduced AP-2 α levels in the nucleus. AP-2 α protein levels have been reported to be lower in the nuclear fraction of astrocytoma samples which was associated with increased malignancy (Britto et al., 2007). In contrast, overexpression of AP-2 α induced cell cycle arrest and apoptosis (Wajapeyee et al., 2006; Wajapeyee and Somasundaram, 2003; Zeng et al., 1997). It has been reported that protein levels of AP-2 α and AP-2 β differed in the nuclear extracts of brain tissue from carbamazepine-treated rats (Rao et al., 2007). In that study, it was suggested that carbamazepine might be differentially affecting cAMP-dependent PKA activity upstream of the AP-2 isoforms. Taken together, these studies suggest that the AP-2 isoforms respond differently under various treatment conditions. Two key questions that remain unanswered are if the cell requires an optimal ratio of AP-2 α /AP-2 β and the mechanism that induces movement of AP-2 α out of the nucleus.

We have shown that A β (42) stimulates apoE mRNA and protein expression in astrocytes. Upregulation of apoE by A β involved β ARs and the transcription factor AP-2 β . It is well-

established that stimulation of β AR increases cAMP levels through activation of Gs protein and adenylate cyclase. A β (42) elevates cAMP levels in mouse primary astrocytes (Igbavboa et al., 2006). Separate lines of evidence showed that cAMP regulates transcription factor AP-2 (Damberg, 2005; Garca et al., 1996) and we observed that cAMP increased AP-2 β levels in the nuclear fraction of astrocytes (data not shown). We now demonstrate that A β (42) increased AP-2 β in the nuclear fraction of astrocytes. The importance of A β -induced AP-2 β in the nucleus is seen by our results showing that cells expressing AP-2 and incubated with A β (42) had enhanced activation of the *apoE* promoter region containing AP-2 binding sites in comparison with AP-2 deficient cells. We propose the following model of A β stimulation of apoE levels which is shown in Figure 4. A β acting on the β AR triggers an increase in cAMP which in turn activates AP-2 resulting in apoE transcription and translation. The newly formed apoE may be secreted and lipidated by ABCA1 (Fan et al., 2011). Consequences of an increase in apoE are not understood and there is evidence that apoE can impair clearance of A β from brain and that such effects are associated with apoE isoform (Deane et al., 2008). In contrast, both apoE and β ARs are thought to play important roles in neuroprotection afforded by astrocytes (Junker et al., 2002; Laureys et al., 2010; Rebeck et al., 2002). There is increasing evidence that apoE mimetics are neuroprotective in models of AD and neuronal injury (Christensen et al., 2011; Li, et al., 2010b; Wang et al., 2007). A β directly binds to the β_2 AR (Wang et al., 2010) and the β_2 AR is upregulated after brain injury (Cleary et al., 1995; Hodges-Savola et al., 1996; Junker et al., 2002). β AR agonists increase apoE protein expression (Cedazo-Mnuez et al., 2001; Igbavboa et al., 2006). A straightforward conclusion is that a combination of apoE mimetics and β_2 AR agonists may have therapeutic utility in regulating astrocyte function in acute brain injury and certain neurodegenerative diseases.

4. Experimental procedures

4.1 Reagents

A β (42) was obtained from American Peptide (Sunnyvale, CA, USA). The concentration and form of A β used were based on our earlier studies showing that soluble or untreated A β (42) increased apoE protein levels, but that aggregated and oligomeric A β (42) did not (Igbavboa et al., 2006; Igbavboa et al., 2003). Furthermore, we have previously reported that soluble A β (42) at the concentration used in the present study consisted mostly of monomers, dimers and trimers, as determined by gel electrophoresis (Igbavboa et al., 2009). One mg of A β (42) was dissolved in 2.3 mL of distilled water containing NH₄OH (1 μ L of 14.8 N ammonium hydroxide in 1 mL of twice-distilled water). The completely solubilized preparation was used immediately without aging. Freshly prepared A β (42) at a concentration of 1 μ M was used for all experiments. The chemicals used, unless specified, were purchased from Sigma (St. Louis, MO, USA). Concentrations of the β AR agonist and antagonists used were based on earlier studies from our lab and others (Baker et al., 2003; Igbavboa et al., 2006): isoproterenol 20 μ M; propranolol 100 μ M; betaxolol 150 μ M; ICI 118551 15 μ M and clenbuterol 15 μ M.

4.2 Cell culture

Primary cortical astrocytes were prepared from 1–2 day old C57BL/6 mice using procedures we have previously reported (Igbavboa et al., 2006; Igbavboa et al., 2009). Cerebral cortices were dissected and meninges removed. Brain tissue samples were kept in cold DMEM (Invitrogen, Carlsbad, CA, USA) containing 10% FBS (Invitrogen, Carlsbad, CA, USA), 1% of PSN Antibiotic Mix (Invitrogen, Carlsbad, CA, USA) and 0.1% of Fungin (InvivoGen, San Diego, CA, USA) and then rinsed with DPBS (Invitrogen, Carlsbad, CA, USA). The tissue was then minced and suspended in a small volume of TrypLE Express (Invitrogen, Carlsbad, CA, USA) for 5–7 min at 37C. The tissue homogenate was

suspended in warm DMEM and filtered through a 70 μ m cell strainer. The cell suspension was then transferred to 25cm² culture flasks (average 1 brain/flask). Media were changed after 24 h and every 2 days thereafter. When cells became confluent (around 7–10 days), they were rinsed with DPBS, suspended in TrypLE Express and subcultured at 1.25×10^6 cells/75 cm² flask. When cells reached approximately 80% confluence, media were replaced with DMEM containing 1% lipoprotein deficient serum (Sigma, St. Louis, MO, USA) and 0.1% PSN Antibiotic Mix. The experiments were performed 24 h after this pre-treatment. All procedures using mice were conducted in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care and the National Institutes of Health policies on the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of the University of Minnesota.

Immortalized DITNC1 rat astrocytes and immortalized HepG2 hepatic cells were purchased from ATCC (Manassas, VA, USA). DITNC1 and HepG2 cells were grown in DMEM with 10% FBS and 1% PSN. For transfection experiments, cells were serum-starved for 24 h before the indicated treatment, unless otherwise noted.

4.3 RT-PCR and agarose gel electrophoresis

After treating cells with A β (42), they were rinsed with DPBS, a small volume of TrypLE Express was added and the cells were scraped from the flasks. TNS solution (Lonza, Basel, Switzerland) was added to stop enzymatic reactions and the cell suspension was collected in a pre-chilled tube. The suspension was centrifuged for 3 min at 2500 rpm, the supernatant removed, the cell pellet resuspended in a small volume of PBS and transferred to a microcentrifuge tube, which was centrifuged again for 2 min at 4000 rpm and the pellet was stored at -20°C , if not used immediately. The cell pellet was homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA was extracted and precipitated following the manufacturer's instructions and resuspended in nuclease-free water (Ambion, Austin, TX, USA). Total RNA was quantified by measuring absorbance at 260 nm.

For the reverse transcription reaction, 0.15 μ g of total RNA was used. RT-PCR was accomplished using the Super Script III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA) and a Bio-Rad icycler thermal cycler (Bio-Rad, Hercules, CA, USA). The program for the thermal cycler, without a hot start, was as follows: cDNA synthesis for 1 cycle at 55°C for 30 min, denaturation for 1 cycle at 94°C for 2 min, PCR amplification for 35 cycles: 94°C for 15 s (denature), 51.3°C for 30 s (anneal), 68°C for 1.5 min (extend), and final extension at 68°C for 5 min, with the finishing cycle at 4°C . The primers used were the following: apoE (503 bp) 5'-AGGATCTACGCAACCGACTC-3', 3'-GGCGATGCATGTCTTCCACTA-5' and β -actin (924 bp) used as an internal standard 5'-GGCCCAGAGCAAGACAGGTA-3' and 3'-GGACTCATCGTACTCCTGCT-5'. The resulting products were electrophoresed on 2% agarose gels with 8% ethidium bromide. The bands were visualized using the Eagle Eye II video system. Band density was quantitated by densitometry using the EagleSight software (Stratagene, La Jolla, CA, USA).

4.4 Real time PCR

Mouse primary astrocytes were incubated with A β (42) (1 μ M) in the presence or absence of the non-selective β AR antagonist propranolol (100 μ M), the β_1 AR selective antagonist betaxolol (150 μ M) or the β_2 AR selective antagonist ICI 118551 (15 μ M) for 60 min. This incubation time was based on the time for maximal A β -induced stimulation of apoE mRNA expression in astrocytes (see Fig. 2A). Antagonists were preincubated with cells for 2 min prior to the addition of A β . Following the different treatments, the RNA isolation protocol was followed as described in the RT-PCR section.

For real time PCR, the iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) was used. RNA was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) following the conditions specified by the manufacturer. To set-up the reaction, we followed the manufacturer's recommendations using 150 ng of cDNA, 0.2 μ M *apoE* primers and 0.05 μ M *GAPDH* primers (reference gene). The sequences for the primer pairs used were: *ApoE*(203bp) 5'-GAGGAACAGACCCAGCAAATA-3', 5'-GTTGTTGCAGGACAGGAGAAG-3' and *GAPDH* (150 bp) 5'-GACATCAAGAAGGTGGTGAAGCAG-3', 5'-AAGGTGGAAGAATGGGAGTTGC-3'. The primers were designed using the Primer 3 program (<http://frodo.wi.mit.edu/primer3/input.htm>). The cycling conditions were: cycle 1: 3 min at 95°C, cycle 2: 10 s at 95°C, 30 s at 56°C for *apoE* and 58°C for *GAPDH*, 1 min at 72°C, all repeat 45 times, cycle 3: 20 s at 55°. The thermal cycler/detection instrument used was the iQ5 (Bio-Rad, Hercules, CA, USA). Data are presented as the means $\pm$ SE for a group of 3 samples. The *apoE* mRNA expression ratio was obtained after normalizing the *apoE* data versus the reference gene (*GAPDH*). The data were analyzed using the Livak Method ($2^{-\Delta\Delta CT}$).

4.4 ApoE and AP-2 protein determination using Western blot analysis

After A β (42) or other treatments, cells were rinsed with cold DPBS and the cells were scraped from the flasks. The cell suspension was collected in a pre-chilled 15 mL tube and centrifuged for 3 min at 1500 rpm. The cell pellet was lysed with complete RIPA buffer (Santa Cruz Biotechnologies, Santa Cruz, CA, USA). The solution was gently rocked for 15 min on ice. The lysate was transferred to a 1.5 mL chilled tube and centrifuged at 13000 rpm for 15 min. The supernatant was transferred to a new tube and protein levels measured. Protein levels were quantitated by measuring the absorbance of a 1:10 dilution of the lysate at 540 nm following the Bradford Protein Assay (Bio-Rad, Hercules, CA, USA) using BSA (Sigma, St. Louis, MO, USA) as a standard.

For Western blot analysis of *apoE*, cell lysates were electrophoresed on a 10% SDS-Tricine-HCl gel (Bio-Rad, Hercules, CA, USA). The proteins were then transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) and incubated with mouse primary monoclonal anti-mouse *apoE* antibody (1:500) from Abcam (Cambridge, UK) and a goat anti-mouse IgG:HRP conjugate (1:5000) was used as a secondary antibody (BD Laboratory, San Diego, CA, USA). Immunoreactivity was visualized with SuperSignal West Pico Chemiluminescent reagent (Pierce, Rockford, IL, USA). Band density was quantitated by densitometry using an Eagle Eye II video system and EagleSight software. Lamin protein was used as a loading control (Santa Cruz Biotechnologies, Santa Cruz, CA, USA).

AP-2 protein levels were determined in cell lysates and nuclear fractions. Extraction of the nuclear fraction was performed using the Nuclear Extraction Kit according to the manufacturer's instructions (Active Motif, Carlsbad, California, USA). Cells were rinsed, collected, lysed as described above and the supernatant (cytoplasmic fraction) was collected by centrifugation, followed by lysis of the leftover pellet and the supernatant (nuclear fraction) was collected by centrifugation. Protein levels were quantitated by measuring the absorbance of a 1:5 dilution of the extract at 540 nm following the Bradford Protein Assay using BSA as a standard. Cell lysates and nuclear extracts were electrophoresed on a 10% SDS-Tris-HCl gel. The proteins were then transferred to a nitrocellulose membrane, blocked and incubated with either rabbit polyclonal (1:1000) or a mouse monoclonal (1:500) antibody specific for AP-2 α or specific for AP-2 β from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat anti-rabbit IgG:HRP conjugate (1:60000; Pierce) or goat anti-mouse IgG:HRP conjugate (1:5000; BD Laboratory) were used as secondary antibodies. Immunoreactivity was visualized with SuperSignal West Pico Chemiluminescent reagent. Band density was quantitated by densitometry using an Eagle Eye II video system and

EagleSight software. Lamin protein was used as a loading control; the LaminA/C rabbit polyclonal antibody (1:200) and goat anti-rabbit IgG:HRP conjugate (1:6000) was used as a secondary antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA).

4.6 Plasmid construction and DNA isolation

The *apoE* sequence (García *et al.*, 1996) of the proximal promoter fragment (Fig. 1) encompassing the 5' region between positions –227 and +400 of the *apoE* gene (construct 4) was used (GeneScript, Piscataway, NJ, USA) for cloning and subcloning. This fragment was subcloned in front of the luciferase reporter gene of the firefly pGL4.12[*luc2CP*] vector (Promega, Madison, WI, USA). The gene product was transferred into bacteria, colonies of the bacterial culture were grown, and a positive clone was isolated and grown on LB medium. The DNA was isolated using the HiSpeed Plasmid Midi Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA values were quantitated by measuring absorbance at 260 nm.

4.7 Transfection and gene reporter assays

DITNC1 and HepG2 cells were co-transfected in 6 well plates with 1 µg of the firefly *apoE-luc* plasmid and 20 ng of *Renilla* pGL4.74[*hRluc*/TK] vector (Promega, Madison, WI, USA) using FuGENE HD reagent (transfection ratio was 3:1; Roche, Basel, Switzerland). Starved cells were treated with Aβ(42) (1 µM). Luciferase assays were performed in triplicate using the Dual-Luciferase Reporter Assay from Promega.

4.8 Statistical analysis

Experimental data are based on results with 3 to 5 independent cell culture preparations. Differences between groups were analyzed using Student's *t* test. Statistical significance was established at a level of $p \leq 0.05$.

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References

1. Baker JG, Hall IP, Hill SJ. Agonist and inverse agonist actions of beta-blockers at the human beta 2-adrenoceptor provide evidence for agonist-directed signaling. *Mol Pharmacol.* 2003; 64:1357–1369. [PubMed: 14645666]
2. Britto R, Umesh S, Hegde AS, Hegde S, Santosh V, Chandramouli BA, Somasundaram K. Shift in AP-2alpha localization characterizes astrocytoma progression. *Cancer Biol Ther.* 2007; 6:413–418. [PubMed: 17471019]
3. Cedazo-Mínguez A. Apolipoprotein E and Alzheimer's disease: Molecular mechanisms and therapeutic opportunities. *J Cell Mol Med.* 2007; 11:1227–1238. [PubMed: 18205697]
4. Cedazo-Mínguez A, Hamker U, Meske V, Veh RW, Hellweg R, Jacobi C, Albert F, Cowburn RF, Ohm TG. Regulation of apolipoprotein E secretion in rat primary hippocampal astrocyte cultures. *Neuroscience.* 2001; 105:651–661. [PubMed: 11516830]
5. Christensen DJ, Ohkubo N, Oddo J, Van Kanegan MJ, Neil JE, Li FQ, Colton CA, Vitek MP. Apolipoprotein E and peptide mimetics modulate inflammation by binding the SET protein and activating protein phosphatase 2A. *J Immunol.* 2011; 186:2535–2542. [PubMed: 21289314]
6. Cleary J, Hittner JM, Semotuk M, Mantyh P, O'Hare E. Beta-amyloid (1–40) effects on behavior and memory. *Brain Res.* 1995; 682:69–74. [PubMed: 7552329]

7. Coelho DJ, Sims DJ, Ruegg PJ, Minn I, Muench AR, Mitchell PJ. Cell type-specific and sexually dimorphic expression of transcription factor AP-2 in the adult mouse brain. *Neuroscience*. 2005; 134:907–919. [PubMed: 16009501]
8. Damberg M. Transcription factor AP-2 and monoaminergic functions in the central nervous system. *J Neural Transm*. 2005; 112:1281–1296. [PubMed: 15959839]
9. Deane R, Sagare A, Hamm K, Parisi M, Lane S, Finn MB, Holtzman DM, Zlokovic BV. ApoE isoform-specific disruption of amyloid beta peptide clearance from mouse brain. *J Clin Invest*. 2008; 118:4002–4013. [PubMed: 19033669]
10. Du Y, Chen X, Wei X, Bales KR, Berg DT, Paul SM, Farlow MR, Maloney B, Ge YW, Lahiri DK. NF-(kappa)B mediates amyloid beta peptide-stimulated activity of the human apolipoprotein E gene promoter in human astroglial cells. *Brain Res Mol Brain Res*. 2005; 136:177–188. [PubMed: 15893602]
11. Eckert D, Buhl S, Weber S, Jäger R, Schorle H. The AP-2 family of transcription factors. *Genome Biol*. 2005; 6:246–246.8. [PubMed: 16420676]
12. Fan J, Donkin J, Wellington CL. Greasing the wheels of Abeta clearance in Alzheimer's disease: the role of lipids and apolipoprotein E. *Biofactors*. 2009; 35:239–248. [PubMed: 19472365]
13. Fan J, Stukas S, Wong C, Chan J, May S, DeValle N, Hirsch-Reinshagen V, Wilkinson A, Oda MN, Wellington CL. An ABCA1-independent pathway for recycling a poorly lipidated 8.1 nm apolipoprotein E particle from glia. *J Lipid Res*. 2011; 52:1605–1616. [PubMed: 21705806]
14. García MA, Campillos M, Marina A, Valdivieso F, Vázquez J. Transcription factor AP-2 is modulated by protein kinase A-mediated phosphorylation. *FEBS Lett*. 1999; 444:27–31. [PubMed: 10037142]
15. García MA, Vázquez J, Giménez C, Valdivieso F, Zafra F. Transcription factor AP-2 regulates human apolipoprotein E gene expression in astrocytoma cells. *J Neurosci*. 1996; 16:7550–7556. [PubMed: 8922411]
16. Göritz C, Mauch DH, Nägler K, Pfrieder FW. Role of glia-derived cholesterol in synaptogenesis: New revelations in the synapse-glia affair. *Journal of Physiology*. 2002; 96:257–263. [PubMed: 12445904]
17. Haasdijk ED, Vluga A, Mulder MT, Jaarsma D. Increased apolipoprotein E expression correlates with the onset of neuronal degeneration in the spinal cord of G93A-SOD1 mice. *Neurosci Lett*. 2002; 335:29–33. [PubMed: 12457735]
18. Hodges-Savola C, Rogers SD, Ghilardi JR, Timm DR, Mantyh PW. Beta-adrenergic receptors regulate astrogliosis and cell proliferation in the central nervous system in vivo. *Glia*. 1996; 17:52–62. [PubMed: 8723842]
19. Hu J, LaDu MJ, Van Eldik LJ. Apolipoprotein E attenuates beta-amyloid-induced astrocyte activation. *J Neurochem*. 1998; 71:1626–1634. [PubMed: 9751196]
20. Igbavboa U, Johnson-Anuna LN, Rossello X, Butterick TA, Sun GY, Wood WG. Amyloid beta-protein 1–42 increases cAMP and apolipoprotein E levels which are inhibited by β 1 and β 2-adrenergic receptor antagonists in mouse primary astrocytes. *Neuroscience*. 2006; 142:655–660. [PubMed: 16904834]
21. Igbavboa U, Pidcock JM, Johnson LNA, Malo TM, Studniski A, Sun GY, Wood WG. Cholesterol distribution in the Golgi complex of DITNC1 astrocytes is differentially altered by fresh and aged amyloid beta-peptide_{1–42}. *J Biol Chem*. 2003; 278:17150–17157. [PubMed: 12584199]
22. Igbavboa U, Sun GY, Weisman GA, Wood WG. Amyloid -protein stimulates trafficking of cholesterol and caveolin-1 from the plasma membrane to the Golgi complex in mouse primary astrocytes. *Neuroscience*. 2009; 162:328–338. [PubMed: 19401218]
23. Ignatius MJ, Gebicke-Harter PJ, Skene JH, Schilling JW, Weisgraber KH, Mahley RW, Shooter EM. Expression of apolipoprotein E during nerve degeneration and regeneration. *Proc Natl Acad Sci U S A*. 1986; 83:1125–1129. [PubMed: 2419900]
24. Junker V, Becker A, Hühne R, Zembatov M, Ravati A, Culmsee C, Kriegelstein J. Stimulation of β -adrenoceptors activates astrocytes and provides neuroprotection. *European Journal of Pharmacology*. 2002; 446:25–36. [PubMed: 12098582]

25. Kimura N, Negishi T, Ishii Y, Kyuwa S, Yoshikawa Y. Astroglial responses against abeta initially occur in cerebral primary cortical cultures: species differences between rat and cynomolgus monkey. *Neurosci Res.* 2004; 49:339–346. [PubMed: 15196782]
26. LaDu MJ, Shah JA, Reardon CA, Getz GS, Bu G, Hu J, Guo L, Van Eldik LJ. Apolipoprotein E receptors mediate the effects of β -amyloid on astrocyte cultures. *J Biol Chem.* 2000; 275:33974–33980. [PubMed: 10940295]
27. LaDu MJ, Shah JA, Reardon CA, Getz GS, Bu G, Hu J, Guo L, Van Eldik LJ. Apolipoprotein E and apolipoprotein E receptors modulate A β -induced glial neuroinflammatory responses. *Neurochem Int.* 2001; 39:427–434. [PubMed: 11578778]
28. Lahiri DK. Apolipoprotein E as a target for developing new therapeutics for Alzheimer's disease based on studies from protein, RNA, and regulatory region of the gene. *J Mol Neurosci.* 2004; 23:225–233. [PubMed: 15181251]
29. Laureys G, Clinckers R, Gerlo S, Spooren A, Wilczak N, Kooijman R, Smolders I, Michhhhotte Y, De Keyser J. Astrocytic beta(2)-adrenergic receptors: from physiology to pathology. *Prog Neurobiol.* 2010; 91:189–199. [PubMed: 20138112]
30. Li FQ, Fowler KA, Neil JE, Colton CA, Vitek MP. An apolipoprotein E-mimetic stimulates axonal regeneration and remyelination after peripheral nerve injury. *J Pharmacol Exp Ther.* 2010a; 334:106–115. [PubMed: 20406857]
31. Li FQ, Fowler KA, Neil JE, Colton CA, Vitek MP. An apolipoprotein E-mimetic stimulates axonal regeneration and remyelination after peripheral nerve injury. *J Pharmacol Exp Ther.* 2010b; 334:106–115. [PubMed: 20406857]
32. Lynch JR, Morgan D, Mance J, Matthew WD, Laskowitz DT. Apolipoprotein E modulates glial activation and the endogenous central nervous system inflammatory response. *J Neuroimmunol.* 2001; 114:107–113. [PubMed: 11240021]
33. Maloney B, Ge YW, Alley GM, Lahiri DK. Important differences between human and mouse APOE gene promoters: Limitation of mouse APOE model in studying Alzheimer's disease. *J Neurochem.* 2007; 103:1237–1257. [PubMed: 17854398]
34. Martinez M, Hernandez AI, Heranz A. Increased cAMP immunostaining in cerebral vessels in Alzheimer's disease. *Brain Res.* 2001; 922:148–152. [PubMed: 11730714]
35. Martinez M, Hernandez AI, Heranz A. Increased cerebrospinal fluid cAMP levels in Alzheimer's disease. *Brain Res.* 2001; 846:265–267. [PubMed: 10556645]
36. Michikawa M, Gong JS, Fan QW, Sawamura N, Yanagisawa K. A novel action of Alzheimer's amyloid β -protein (A β): Oligomeric A β promotes lipid release. *J Neurosci.* 2001; 18:7226–7235. [PubMed: 11549733]
37. Moser M, Imhof A, Pscherer A, Bauer R, Amselgruber W, Sinowatz F, Hofstädter F, Schüle R, Buettner R. Cloning and characterization of a second AP-2 transcription factor: AP-2 beta. *Development.* 1995; 121:2779–2788. [PubMed: 7555706]
38. Oulad-Abdelghani M, Bouillet P, Chazaud C, Dollé P, Chambon P. AP2.2: A novel AP-2 related transcription factor induced by retinoic acid during differentiation of P19 embryonal carcinoma cells. *Exp Cell Res.* 1996; 225:338–347. [PubMed: 8660922]
39. Oyama F, Shimada H, Oyama R, Ihara Y. Apolipoprotein E genotype, Alzheimer's pathologies and related gene expression in the aged population. *Brain Res Mol Brain Res.* 1995; 29:92–98. [PubMed: 7770005]
40. Poirier J, Hess M, May PC, Finch CE. Astrocytic apolipoprotein E mRNA and GFAP mRNA in hippocampus after entorhinal cortex lesioning. *Brain Res Mol Brain Res.* 1991; 11:97–106. [PubMed: 1661818]
41. Praopong T, Uemura E, Hsu WH. G protein and cAMP-dependent protein kinase mediate amyloid β -peptide inhibition of neuronal glucose uptake. *Exp Neurol.* 2001; 167:59–64. [PubMed: 11161593]
42. Rao JS, Bazinet RP, Rapoport SI, Lee HJ. Chronic administration of carbamazepine down-regulates AP-2 DNA-binding activity and AP-2 α protein expression in rat frontal cortex. *Biol Psychiatry.* 2007; 61:154–161. [PubMed: 16806101]
43. Rebeck GW, Kindy M, LaDu MJ. Apolipoprotein E and Alzheimer's disease: The protective effects of ApoE2 and E3. *J Alzheimers Dis.* 2002; 4:145–154. [PubMed: 12226533]

44. Seitz A, Kragol M, Aglow E, Showe L, Heber-Katz E. Apolipoprotein E expression after spinal cord injury in the mouse. *J Neurosci Res.* 2003; 71:417–426. [PubMed: 12526030]
45. Shimada M, Konishi Y, Ohwawa N, Ohtaka-Maruyama C, Hanaoka F, Makino Y, Tamura T. Distribution of AP-2 subtypes in the adult mouse brain. *Neurosci Res.* 1999; 33:275–280. [PubMed: 10401980]
46. Vance JE, Hayashi H, Karten B. Cholesterol homeostasis in neurons and glial cells. *Sem Cell Dev Biol.* 2005; 16:193–212.
47. Wajapeyee N, Britto R, Ravishankar HM, Somasundaram K. Apoptosis induction by activator protein 2alpha involves transcriptional repression of Bcl-2. *J Biol Chem.* 2006; 281:16207–16219. [PubMed: 16533807]
48. Wajapeyee N, Somasundaram K. Cell cycle arrest and apoptosis induction by activator protein 2alpha (AP-2alpha) and the role of p53 and p21 WAF1/CIP1 in AP-2alpha-mediated growth inhibition. *J Biol Chem.* 2003; 278:52093–52101. [PubMed: 14551210]
49. Wang D, Govindaiah G, Liu R, De Arcangelis V, Cox CL, Xiang YK. Binding of amyloid beta peptide to beta2 adrenergic receptor induces PKA-dependent AMPA receptor hyperactivity. *FASEB J.* 2010; 24:3511–3521. [PubMed: 20395454]
50. Wang D, Yuen EY, Zhou Y, Yan Z, Xiang YK. Amyloid beta peptide-(1–42) induces internalization and degradation of beta(2) adrenergic receptors in prefrontal cortical neurons. *J Biol Chem.* 2011; 286:31852–31863. [PubMed: 21757762]
51. Wang H, Durham L, Dawson H, Song P, Warner DS, Sullivan PM, Vitek MP, Laskowitz DT. An apolipoprotein E-based therapeutic improves outcome and reduces Alzheimer's disease pathology following closed head injury: Evidence of pharmacogenomic interaction. *Neuroscience.* 2007; 144:1324–1333. [PubMed: 17187933]
52. Yamada T, Kondo A, Takamatsu J, Tateishi J, Goto I. Apolipoprotein E mRNA in the brains of patients with Alzheimer's disease. *J Neurol Sci.* 1995; 129:56–61. [PubMed: 7751846]
53. Yamagata K, Urakami K, Ikeda K, Ji Y, Adachi Y, Arai H, Sasaki H, Sato K, Nakashima K. High expression of apolipoprotein E mRNA in the brains with sporadic Alzheimer's disease. *Dement Geriatr Cogn Disord.* 2001; 12:57–62. [PubMed: 11173875]
54. Zarow C, Victoroff J. Increased apolipoprotein E mRNA in the hippocampus in Alzheimer disease and in rats after entorhinal cortex lesioning. *Exp Neurol.* 1998; 149:79–86. [PubMed: 9454617]
55. Zeng YX, Somasundaram K, El-Deiry WS. AP2 inhibits cancer cell growth and activates p21WAF1/CIP1 expression. *Nat Genet.* 1997; 15:78–82. [PubMed: 8988173]

Highlights

1. Amyloid beta-protein stimulates apoE mRNA expression and protein levels in mouse primary astrocytes.
2. Effects of A β on apoE are mediated by the β_2 -adrenergic receptor and the transcription factor AP-2.
3. A β increases AP-2 β in a nuclear fraction which binds to the apoE promoter.
4. Transcriptional upregulation of apoE expression by A β may be a neuroprotective response to A β -induced cytotoxicity, consistent with apoE's role in cytoprotection

Fig. 1. Aβ(42) increases apoE mRNA and protein levels in mouse primary astrocytes

A. mRNA levels at different time points. Cells were incubated with Aβ(42) (1 μM) for 15, 30, 60, 90 or 120 minutes. Levels of apoE mRNA were determined by RT-PCR and quantitated by densitometry in agarose gels; n = 3; * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0005$. Data were normalized using β-actin mRNA whose expression level was unaffected by Aβ. Data are from 3 independent experiments for each time point. **B. ApoE protein levels.** Cells were incubated with Aβ₁₋₄₂ (1 μM) for 60 and 120 minutes. Levels of apoE protein were determined by Western blot and quantified by densitometry. The data was normalized in respect to lamin protein levels (apoE:lamin C ratio) and presented as the percentage increase from control. n = 4. * $p \leq 0.01$, ** $p \leq 0.001$. **C. βAR antagonists and mRNA levels.** Cells were incubated with Aβ(42) (1 μM) and propranolol (PR, 100 μM), betaxolol (BTX, 150 μM) or ICI 118551 (ICI, 15 μM) for 60 min. Incubation conditions and drug concentrations were the same as previously reported for effects of Aβ(42) on apoE protein levels ((Igbavboa *et al.*, 2006). Levels of apoE mRNA were determined by real time PCR and normalized against the housekeeping gene GAPDH using the Livak method ($2^{-\Delta\Delta CT}$); n = 3; # $p \leq 0.005$ (versus Control group), * $p \leq 0.01$, ** $p \leq 0.005$ (versus Aβ group). Data are from 3 independent experiments for each treatment condition.

Fig. 2. Aβ(42) effects on AP-2α and β protein levels in the nuclear fraction and cell lysate in mouse primary astrocytes

Cells were incubated with soluble Aβ(42) (1 μM) for 10 min. Extraction of the nuclear fraction was performed using the Nuclear Extraction Kit according to the manufacturer's instructions (Active Motif, Carlsbad, California, USA). Protein levels were determined by Western blot analysis. Data are means ± S.E. of the densitometric scans from Western blots representing AP-2 immunoreactivity (n = 6); * $p \leq 0.00001$. Insets are Western blots of representative experiments. Data are from 3 independent experiments. **A. AP-2α Nuclear Fraction. B. AP-2β Nuclear Fraction. C. AP-2α Cell lysate. D. AP-2β Cell Lysate.**

Fig 3. Aβ(42) stimulates apoE promoter activity

DITNC1 and HepG2 cells were co-transfected with a vector containing the firefly luciferase reporter gene (pGL4.12[*luc2CP*]) downstream of a fragment of the apoE promoter with functional AP-2 binding sites and a *Renilla* vector, then treated with Aβ(42). Dual luciferase activities were determined and results expressed as a percentage increase over untreated control cells after normalization with the *Renilla* values. Values are the means ± S.E. (n=3); ** $p \leq 0.01$. Data are from 3 independent experiments for each treatment condition.

Fig. 4. Proposed model of Aβ-induced stimulation of apoE abundance in astrocytes

Aβ binds to the βAR resulting in activation of G_s protein, AC and formation of cAMP. The increase in cAMP stimulates AP-2 activity and binding to AP-2 binding sites on the *apoE* promoter resulting in an increase in apoE protein abundance. Newly synthesized apoE may be secreted and lipidated by ABCA1 where it complexes with members of the low density lipoprotein receptor family.