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EGCG functions through estrogen receptor-mediated activation of ADAM10 in the promotion of non-amyloidogenic processing of APP

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ABSTRACT

Estrogen depletion following menopause has been correlated with an increased risk of developing Alzheimer's disease (AD). We previously explored the beneficial effect of (−)-epigallocatechin-3-gallate (EGCG) on AD mice and found increased non-amyloidogenic processing of amyloid precursor protein (APP) through the α-secretase a disintegrin and metallopeptidase domain 10 (ADAM10). Our results in this study suggest that EGCG-mediated enhancement of non-amyloidogenic processing of APP is mediated by the maturation of ADAM10 via an estrogen receptor-α (ERα)/phosphoinositide 3-kinase/Ak-transforming dependent mechanism, independent of furin-mediated ADAM10 activation. These data support prior assertions that central selective ER modulation could be a therapeutic target for AD and support the use of EGCG as a well-tolerated alternative to estrogen therapy in the prophylaxis and treatment of this disease.

1. Introduction

Over the past decade, intense focus has been given to investigating amyloid precursor protein (APP) processing and β-amyloid (Aβ) metabolism as potential therapeutic targets for Alzheimer's disease (AD) [1]. More recently, attention has turned toward the α-secretase/non-amyloidogenic pathway of APP metabolism [2,3], although its role in AD and potential as a diagnostic marker have been considered for some time [4–7]. Because of the limited amount of APP in the cell, it is believed that the amyloidogenic and non-amyloidogenic pathways compete for substrate in the process of APP proteolysis [8]. Since α-secretase cleaves within the Aβ peptide domain, its activation has the added advantage of precluding neurotoxic Aβ peptide formation.

According to prevalence studies, women have a higher risk of developing AD than men [9,10]. Following menopause, this increased risk of developing AD can be partially attributed to estrogen depletion [11]. In vitro, 17β-estradiol is associated with accumulation of a soluble fragment of APP resulting from soluble amyloid precursor protein alpha (sAPPα) [12] and reduced Aβ generation [13]. In vivo, selective estrogen receptor (ER) modulators reduce Aβ accumulation and improve behavioral performance [14,15]. Despite these promising results, the efficacy of hormone replacement therapy (HRT) in preventing AD in women has remained controversial [16,17]. While some report that postmenopausal women taking HRT have both a decreased risk and delayed onset of developing AD [18], others have found that HRT may result in an increased dementia risk; either directly or due to an elevation of other risk factors [10,19]. Given this debate, the fact that APP processing, ER activity, and the risk of AD are interrelated is not surprising (reviewed by Gandy [20]). Because of this, investigators have attempted to explain the mechanistic underpinnings by which estrogen-mediated signaling affects Aβ accumulation (reviewed by Gandy and Petanceska [21] and Pike et al. [22]).

Abbreviations: EGCG, epigallocatechin-3-gallate; ER, estrogen receptor; PI3K, phosphoinositide 3-kinase; PIP2, phosphatidylinositol [3,4]-bisphosphate; PIP3, phosphatidylinositol [3,5]-triphosphate; AKT, Ak-transforming; p85, ~85 kD regulatory subunit of PI3K; Shc, Src homology 2 domain-containing; APP, amyloid precursor protein; ADAM10, a disintegrin and metalloprotease domain 10; sAPPα, soluble amyloid precursor protein alpha; α-CTF, alpha carboxyl terminal fragment; HRT, hormone replacement therapy; Aβ, β-amyloid

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Green tea compounds have been analyzed for their efficacy in the modulation of APP processing. Arguably one of the most promising green tea compounds being studied is (−)-epigallocatechin-3-gallate (EGCG), which has gained increasing attention due in part to its reported anti-carcinogenic effects [23,24]. One theory is that EGCG may act on the ER via its gallate group, thereby mimicking the 7α-position of 17β-estradiol [25]. Previous reports suggest that EGCG regulates the production of sAPPα through modulation of protein tyrosine phosphatases [26,27] and protein kinase C-dependent mechanisms [28,29]. Additionally, EGCG has been shown to inhibit the activities of pro-inflammatory cytokines [30–32] and a multitude of cellular signaling pathways [31,33,34]; including those involving the phosphatidylinositol 3′-OH kinase (PI3K)/Ak-transforming (Akt) cascade [35].

We have previously shown that EGCG reduces Aβ generation in N2a cells overexpressing Swedish mutant APP (SweAPP N2a) [36]. In concert with these observations, we found that EGCG promotes α-site cleavage of APP to enhance formation of α-carboxyl terminal fragment of APP and sAPPα. These events are associated with elevated α-secretase cleavage activity and enhanced activation of a disintegrin and metalloprotease domain 10 (ADAM10) [37].

In an effort to further characterize the manner in which stimulation of the non-amyloidogenic/α-secretase pathway leads to reductions in Aβ, our current investigation focuses on mechanisms by which EGCG alters APP processing. In the present study, we show that EGCG promotes α-secretase-mediated APP metabolism through both ERα and furin dependent mechanisms. Specifically, EGCG enhanced maturation of ADAM10 via an ERα/PI3K/Akt dependent mechanism, distinct from EGCG-mediated furin upregulation.

2. Materials and methods

2.1. Reagents

Green tea-derived EGCG (95% purity by HPLC) was purchased from Sigma Chemical Co. (St. Louis, Missouri), wortmannin (PI3K inhibitor) was obtained from Calbiochem (San Diego, CA, USA), and the highly selective cell permeable PI3K inhibitor, LY294002, was purchased from Sigma. The selective ERα agonist 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT) was obtained from Sigma and the selective ERα antagonist methyl-piperidino-pyrazole (MPP) and selective ERβ antagonist 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyri midin-3-yl]phenol (PHTPP) were purchased from Tocris Bioscience (Ellisville, MO). The Akt inhibitor triciribine hydrate (TCN), was obtained from Sigma.

2.2. ELISA

Conditioned media were collected and analyzed at a 1:1 dilution using the method as previously described [38] and values were reported as percentage of $A\beta_{1-x}$ secreted relative to control. Quantitation of total Aβ species was performed according to published methods [31]. Briefly, 6E10 (capture antibody) was coated at 2 μg/ml in PBS into 96-well immunoassay plates overnight at 4 °C. The plates were washed with 0.05% Tween-20 in PBS five times and blocked with blocking buffer (PBS with 1% BSA, 5% horse serum) for 2 h at room temperature. Conditioned medium or Aβ standards were added to the plates and incubated overnight at 4 °C. Following 3 washes, biotinylated antibody, 4G8 (0.5 μg/ml in PBS with 1% BSA) was added to the plates and incubated for 2 h at room temperature. After five washes, streptavidin-horseradish peroxidase (1:200 dilutions in PBS with 1% BSA) was added to the 96-wells for 30 min at room temperature. Tetramethylbenzidine (TMB) substrate was added to the plates and incubated for 15 min at room temperature. 50 μl of stop solution (2 N N_2SO_4) was added to each well of the plates. The optical density of each well was immediately determined by a microplate reader at 450 nm. In addition, $A\beta_{1-40}$, or $A\beta_{1-42}$ was separately quantified in these samples using the $A\beta_{1-40}$ $A\beta_{1-42}$ ELISA kits (IBL-America, Minneapolis, MN) in accordance with the manufacturer's instructions. In all cases, Aβ levels were expressed as a percentage of control (i.e., conditioned medium from untreated SweAPP N2a cells).

2.3. Western blot

Cultured cells were lysed in ice-cold lysis buffer described above, and an aliquot corresponding to 20–50 μg of total protein was electrophoretically separated using 10% or 12% Tris–glycine gels. Electrophoresed proteins were then transferred to PVDF membranes (Bio-Rad, Richmond, California), washed in ddH_2O, and blocked for 1 h at ambient temperature in Tris-buffered saline (TBS; Bio-Rad) containing 5% (w/v) non-fat dry milk. After blocking, membranes were hybridized for 1 h at ambient temperature with various primary antibodies. Membranes were then washed three times for 5 min each in ddH_2O and incubated for 1 h at ambient temperature with the appropriate HRP-conjugated secondary antibody (1:1000, Pierce Biotechnology, Inc., Rockford, Illinois). All antibodies were diluted in TBS containing 5% (w/v) of non-fat dry milk. Blots were developed using the luminol reagent (Pierce Biotechnology). Densitometric analysis was done using the Fluor-S MultiImager™ with Quantity One™ software (Bio-Rad). For examining sAPPα, conditioned medium was collected following treatment according to a modified protocol from Chen and Fernandez [39]. sAPPα was extracted using 3 K Nanosep centrifugal filters (Pall Life Sciences, Ann Arbor, Michigan) and protein concentrate was prepared for the aforementioned electrophoresis. Antibodies used for western blot included: ADAM10 antibodies (1:1000; Calbiochem and Chemicon), Furin antibody (1:1000; Biomol Intl., Plymouth Meeting, PA), PC7 antibodies (1:1000; Abcam, Cambridge, MA), phospho-Tyr p85 PI3K binding motif, phospho-Akt (Ser 473), total Akt antibodies (1:1000, Cell Signaling Technology, Danvers, MA, USA), or actin antibody (1:1500; as an internal reference control; Roche).

Densitometric analysis was conducted using the Fluor-S Multi-Imager with Quantity One software (Bio-Rad) or ImageJ software (NIH). Images were scanned, protein bands were captured, and a threshold optical density was obtained that discriminated bands from background. Densitometric values were reported as area of positive pixels in reference to an internal control.

2.4. Statistical analysis

All data were normally distributed; therefore, in instances of single mean comparisons, Levene's test for equality of variances followed by *t*-test for independent samples was used to assess significance. In instances of multiple mean comparisons, analysis of variance (ANOVA) was used, followed by *post hoc* comparison using Bonferonni's method as appropriate. Alpha levels were set at 0.05 for all analyses. The statistical package for the social sciences release 18 (SPSS Inc., Chicago, Illinois) or StatPlus®:mac (AnalystSoft, Inc., Vancouver, British Columbia, CA) was used for all data analysis.

3. Results

The presence of estrogen enhances non-amyloidogenic APP α-secretase cleavage as evidenced by increased sAPPα and decreased Aβ production [40,41]. Thus, the selective actions of estrogen may represent a therapeutic target for the prevention of toxic Aβ species and subsequent neurodegeneration. Our prior investigations suggest a similar mechanism may be involved in EGCG's promotion of sAPPα production [36]. Taken together with studies that

implicate ER modulation after treatment with EGCG [42,43,25], we set out to explore whether EGCG could act fully or partially through the ER to exert its effect on APP processing.

3.1. Estrogen receptor (ERα) inhibition mitigates EGCG-induced ADAM10 activation and non-amyloidogenic APP processing in SweAPP N2a cells

Using similar conditions as in our prior investigations, APP (Swedish mutant APP 695aa isoform) overexpressing murine neuroblastoma N2a cells (SweAPP N2a), known to primarily express ERα [44], were treated with EGCG at 20 μM [36] in the presence of various concentrations (0–2.5 μM and 50–200 μM) of highly selective ERα antagonist MPP [45,46] or highly selective ERβ antagonist PHTPP, as a structurally related 'control compound,' for 12 h (Fig. 1). $A\beta_{1-40,42}$ peptides were analyzed in conditioned media from these cells by ELISA. Consistent with our central hypothesis, data reveal significant increases in $A\beta_{1-40,42}$ peptide production by greater than 100% with co-treatment with EGCG and MPP compared to treatment with EGCG alone. No significant ($P > 0.05$) changes in Aβ peptide production were found with control compound and EGCG cotreatment, thereby suggesting that EGCG promotes non-amyloidogenic processing though ERα modulation.

As our prior investigations demonstrated the requirement of ADAM10 in EGCG promotion of non-amyloidogenic APP metabolism [37], cell lysates from the same SweAPP N2a cells were prepared and subjected to western analysis for ADAM10. Densitometry ratios of mature (mADAM10) to the proform of ADAM10 (pro-ADAM10) band densities at various doses of MPP or control compound treated SweAPP N2a cells show that MPP, but not control compound, significantly inhibits ADAM10 maturation by greater than 70% (Fig. 1). This effect of ERα antagonism correlates with increased production of Aβ peptides thereby indicating downregulation of non-amyloidogenic APP processing.

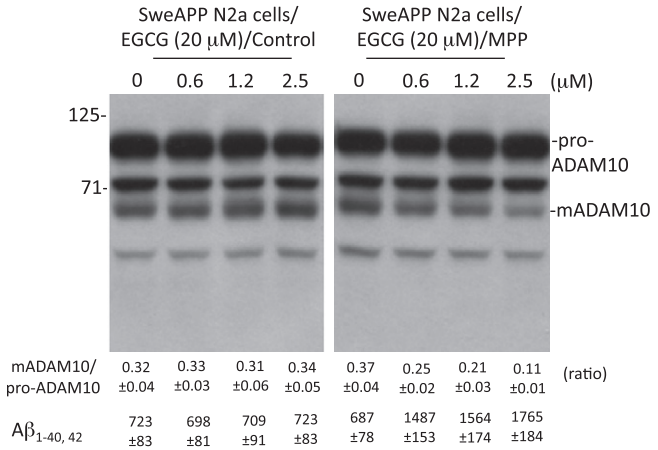

Fig. 1. Estrogen receptor (ERα) inhibition mitigates EGCG-induced ADAM10 activation and non-amyloidogenic APP processing in SweAPP N2a cells. SweAPP N2a cells (murine neuroblastoma cells overexpressing Swedish mutant 695aa isoform of APP) were treated with EGCG at 20 μM in the presence of estrogen inhibitor (MPP, an antagonist at ERα receptor displaying >200-fold selectivity for ERα over ERβ) or a control compound lacking estrogen receptor modulation properties at various doses as indicated for 12 h. $A\beta_{1-40,42}$ peptides were analyzed in conditioned media from these cells by ELISA. Data are represented as $A\beta_{1-40,42}$ (pg) in total cellular protein (mg) secreted 12 h after co-treatment as indicated below the figure. Cell lysates were prepared and subjected to western analysis of ADAM10 maturation. Densitometric analysis shows the ratio of active mature (mADAM10) to proform (pro-ADAM10) as indicated below the figure. One-way ANOVA followed by *post hoc* comparison revealed significant differences between MPP doses (**$P < 0.005$ with $n = 3$ for each condition), but not control inhibitor ($P > 0.05$), for Aβ generation and ratio of mADAM10 to pro-ADAM10.

Conversely, highly selective ERα agonist, PPT (50–200 μM), was utilized to compare the effects of ERα activation on downstream signaling and ADAM10 maturation with the previously observed effects of EGCG. SweAPP N2a cells were treated for 12 h. Similar to EGCG, western analysis for ADAM10 revealed enhanced ADAM10 maturation with PPT although this effect did not reach statistical significance (Supplementary Fig. 1). Interestingly, PPT and EGCG revealed no additive maturation of ADAM10 possibly secondary to ERα saturation at trialed doses of these compounds. Moreover, PPT treatment seemed to attenuate ERα stimulation by EGCG, perhaps alluding to some form of competition between the two agonists. (Supplementary Fig. 1). However, taken together with the MPP results, these results suggest that mechanistically, EGCG promotion of non-amyloidogenic APP processing mediated by ADAM10 may require the activity of ERα in these cells.

3.2. EGCG failed to directly promote ADAM10 activation in broken cell preparations

Having shown that EGCG markedly enhances ADAM10 maturation via ERα-mediated signaling in SweAPP N2a cells, we next set out to characterize downstream pathway effectors. We hypothesized that EGCG promotion of non-amyloidogenic APP processing requires structured cellular functions, such as indirect signal transduction cascades or gene regulation, rather than direct activation of ADAM10 by EGCG. To help rule out the possibility of direct activation, broken cell preparations from untreated SweAPP N2a cells were treated with EGCG (10 μM) or PBS (Fig. 2). One hour later, these cell lysates were subjected to western analysis for ADAM10. Densitometric analysis indicates that ratios of mADAM10 to pro-ADAM10 did not vary significantly ($P > 0.05$) suggesting that ADAM10 activation by EGCG is not mediated through a direct cytosolic or molecular interaction with EGCG. These findings provide evidence of the requirement for signal transduction pathways and/or gene regulation in EGCG-mediated ADAM10 activation.

3.3. PI3K/Akt signaling is involved in EGCG-mediated ADAM10 activation and promotion of non-amyloidogenic processing of APP

The involvement of PI3K signaling in the non-genomic activities of ERs and related downstream signaling events is well known.

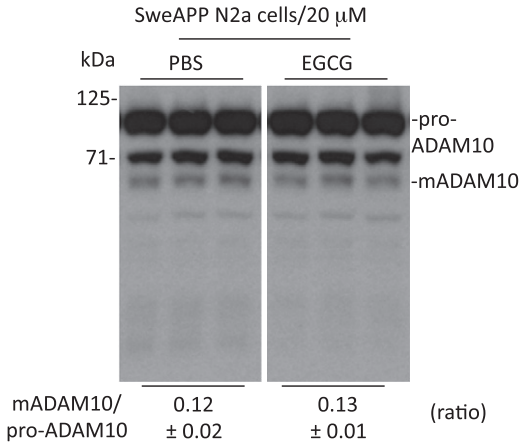

Fig. 2. EGCG failed to directly promote ADAM10 activation in broken SweAPP N2a cell preparations. Cell lysates from untreated SweAPP N2a cells and subsequently treated these lysates with EGCG (10 μM) or PBS. One hour later, these cell lysates were subjected to western analysis of ADAM10. Densitometric analysis shows the ratio of mADAM10 to pro-ADAM10 as indicated below the figure. One-way ANOVA followed by *post hoc* comparison revealed no significant differences between the treated conditions ($P > 0.05$ with $n = 4$ for each condition) for the ratio of mADAM10 to pro-ADAM10.

Comparatively, EGCG is also known to activate PI3K in various cell types [35,47]. Gandy and colleagues had previously supported a role for PI3K activation in promoting sAPPα release from SweAPP N2a cells [48]. Thus, we next investigated the contribution of PI3K to EGCG-mediated ADAM10 activation in SweAPP N2a cells. As shown in Fig. 3, SweAPP N2a cells were treated with EGCG (20 μM) for 12 h and sAPPα secretion was quantified by ELISA after varying treatment concentrations (0–50 μM) of PI3K inhibitor (wortmannin). Our data show that the PI3K inhibition results in a dose-dependent decrease in sAPPα release by SweAPP N2a cells suggesting inhibition of α-secretase activity (Fig. 3A). LDH release did not vary between control treatment doses of wortmannin (data not shown). Based on these findings we hypothesized that PI3K activation regulates ADAM10 activation in these cells.

In order to test this hypothesis, SweAPP N2a cells were treated for 12 h with 20 μM EGCG and varying doses of the PI3K inhibitors, wortmannin (0.2–10 μM) and LY294002 (10–100 μM) [49], lysates were prepared and subjected to western analysis for ADAM10 (Fig. 3B). Consistent with our sAPPα ELISA findings, ratios of mADAM10/pro-ADAM10 show a decrease following treatment with both PI3K inhibitors; suggesting likely involvement of PI3K in EGCG-mediated ADAM10 activation.

As PI3K has multiple downstream effectors, we next analyzed phosphorylation of PI3K's regulatory subunit ~85 kD regulatory subunit of PI3K (p85) in the context of increasing doses of EGCG. SweAPP N2a cells were treated with varying concentration of EGCG (0–40 μM) for 4 h, lysed then subjected to western analysis for PI3K-phospho-p85. Our data indicate a dramatic dose-dependent increase in phosphorylation at this principle activation site with EGCG treatments above 5 μM (Fig. 3C and D), altogether suggesting EGCG promotion of ADAM10 activation and non-amyloidogenic APP processing involves active PI3K signaling.

To further characterize downstream effectors involved in EGCG-mediated ADAM10 activation, we examined Akt and phospho-Akt expression following treatment of SweAPP N2a cells with PI3K inhibitors (wortmannin and LY294002) in the presence of 20 μM EGCG after 4 h. Importantly phospho-Akt increased with EGCG (20 μM) as compared to untreated SweAPP N2a cells, whereas

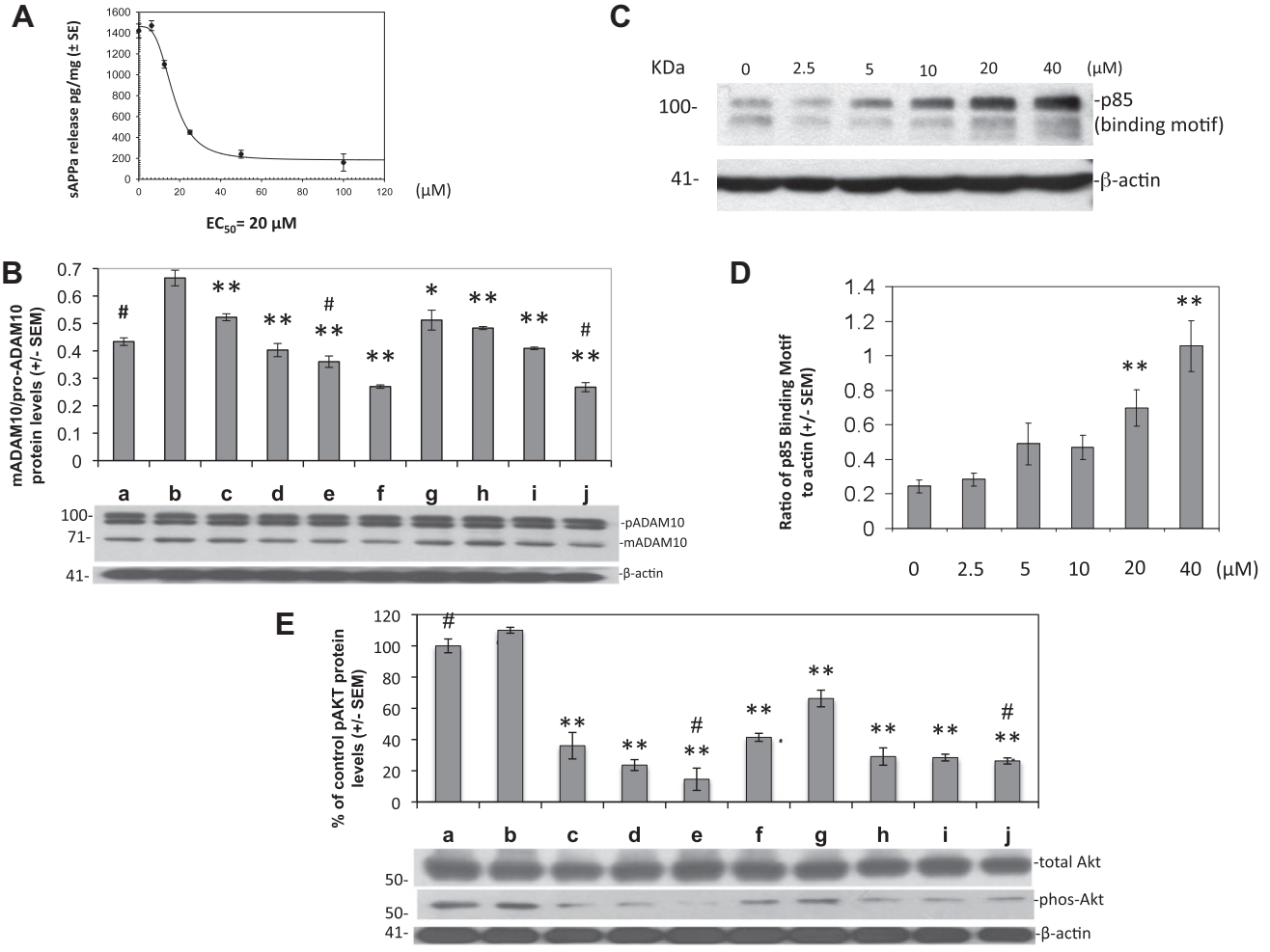

Fig. 3. PI3K/Akt signaling is involved in EGCG-mediated ADAM10 activation and promotion of non-amyloidogenic processing of APP. Various treatment conditions in SweAPP N2a cells are denoted as a–j and correspond to the following: (a) no treatment, (b) EGCG, (c) wortmannin (WM) 200 nM + EGCG, (d) WM 400 nM + EGCG, (e) WM 10 μM + EGCG, (f) WM 400 nM, (g) LY294002 10 μM + EGCG, (h) LY294002 50 μM + EGCG, (i) LY294002 100 μM + EGCG, (j) LY294002 50 μM. EGCG was used at a concentration of 20 μM for all conditions unless otherwise indicated. (A) SweAPP N2a cells were treated with EGCG and sAPPα release was quantitated by ELISA after varying treatment concentrations of PI3K inhibitor (WM). (B) pro-ADAM10 and mADAM10 following treatment with the PI3K inhibitors, WM and LY294002 were analyzed in cell lysates from SweAPP N2a cells by western blot. Densitometry analysis results are represented as band density ratio means ± S.E.M. ($n = 3$), $^{*}P < 0.05$, $^{**}P < 0.01$ of protein of interest compared to EGCG control, # represents the protein of interest compared to control without EGCG treatment. (C and D) SweAPP N2a cell lysates were collected following treatment with various concentrations of EGCG as indicated and analyzed by western analysis for the p85 binding motif of PI3K. Densitometry analysis results are represented as band density ratio of p85 to β-actin (±S.E.M., $n = 3$, $^{**}P < 0.01$). (E) Total Akt and phospho-Akt were assessed as percent of associated control following treatment with the PI3K inhibitors and were analyzed in cell lysates from SweAPP N2a cells by western blot. Densitometry analysis results are represented as band density means ± S.E.M. ($n = 3$), $^{**}P < 0.01$ of protein of interest compared to EGCG control, # represents the protein of interest compared to control without EGCG treatment.

the addition of PI3K inhibitors, dose-dependently reduced phospho-Akt (Fig. 3E). Significant group differences were not observed between total Akt or actin (data not shown). However, in similar treatment conditions, the Akt inhibitor, TCN, was able to decrease ADAM10 activation, total and phospho-Akt, but not actin, in the presence of EGCG (Fig. 4). These data culminate to support the hypothesis that EGCG may effect ADAM10 activation, and subsequent non-amyloidogenic processing of APP, via involvement of ERα/PI3K/Akt dependent signaling mechanisms.

3.4. EGCG enhances the ADAM10 activating enzyme furin independent of PI3K activation in SweAPP N2a cells

ADAM10 is known to be regulated by proprotein convertases, especially PC7 and furin [50]. To determine whether EGCG enhances activation of ADAM10 through the upstream regulation of proprotein convertases, SweAPP N2a cells were treated with varying concentrations of EGCG (0–40 μM) for 4 h, lysed and subjected to western analysis for furin or PC7. Results indicated that expression of furin but not PC7 isoforms were increased nearly 4–5-fold compared with controls (Fig. 5A–D).

Regulation of furin is complex and appears to involve both adaptor proteins and autoactivation depending on the environment of its cellular compartment [51], however, given our findings of PI3K/Akt involvement in EGCG promotion of ADAM10 activation via ERα, we explored the ability of varying doses of PI3K inhibitor to affect EGCG-mediated furin upregulation. SweAPP N2a cells were treated with EGCG (20 μM) in the presence of PI3K inhibitor (wortmannin; 0–50 μM) for 4 h, lysates were prepared and analyzed by western blot (Fig. 5E and F). Interestingly, PI3K inhibition failed to inhibit EGCG-associated furin activation; implicating a divergent, non-PI3K-mediated pathway for EGCG-induced furin activation.

To investigate whether EGCG-mediated furin upregulation could result from non-PI3K dependent activities of ERα, SweAPP N2a cells were treated for overnight in the presence of EGCG (20 μM) and either ERα antagonist (MPP) or agonist (PPT) (Supplementary Fig. 1). Results showed no significant changes in furin protein levels between EGCG and either MPP or PPT thereby altogether implicating an alternative ERα- and PI3K-independent mechanism associated with EGCG-induced furin upregulation.

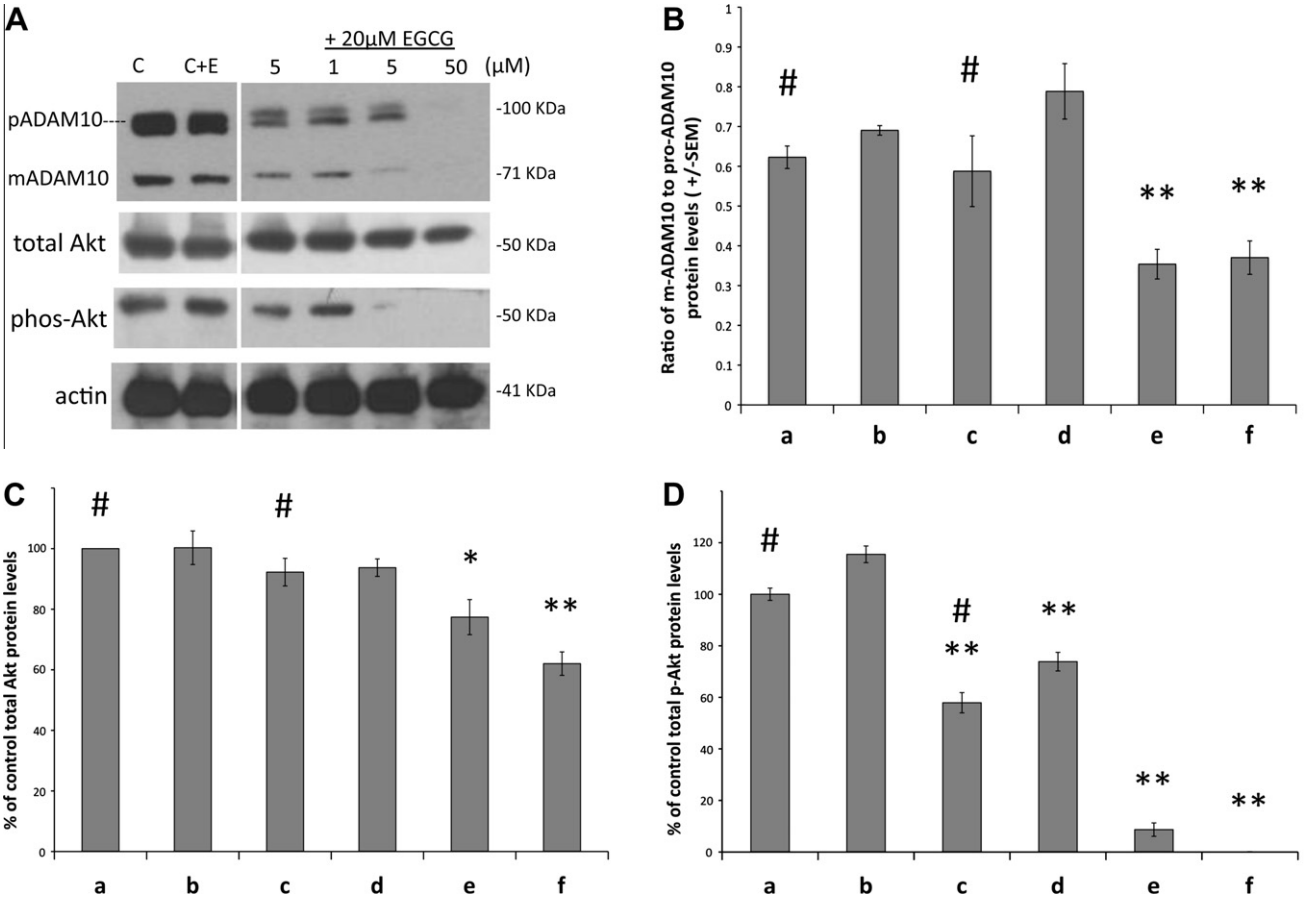

Fig. 4. Akt inhibition by triciribine hydrate (TCN) reduces ADAM10 activation, total Akt, and phosphorylated Akt, in the presence of EGCG. Various treatment conditions in SweAPP N2a cells are denoted as (a–f) and correspond to the following: (a) no treatment, (b) EGCG, (c) TCN 5 μM, (d) TCN 1 μM + EGCG, (e) TCN 5 μM + EGCG, (f) TCN 50 μM + EGCG. EGCG was used at a concentration of 20 μM for all conditions unless otherwise indicated. (A) SweAPP N2a cells were treated with varying concentrations of Akt inhibitor (TCN) in the presence and absence of EGCG. Cell lysates were prepared and subjected to western analysis of ADAM10 maturation, total Akt, phospho-Akt (Ser473), and β-actin (internal control). (B) Densitometric analysis shows the ratio of active mature (mADAM10) to proform (pro-ADAM10) as indicated below the figure. One-way ANOVA revealed significant differences between TCN treated and control cells at concentrations of 5 and 10 μM (**$P < 0.01$ with $n = 3$ for each condition) in the presence of EGCG. # represents the protein of interest compared to control without EGCG treatment. (C and D) Densitometric analysis was performed on total Akt and phospho-Akt (Ser473) and represented as percent of associated control (SweAPP N2a ± EGCG) following treatment with TCN ± S.E.M. ($n = 3$), *$P < 0.05$ and **$P < 0.01$ of protein of interest compared to EGCG control, # represents the protein of interest compared to control without EGCG treatment. Significant differences between treated and control were present at concentrations of 5 and 10 μM for total Akt in the presence of EGCG only, while phospo-Akt was significantly reduced for all TCN concentrations in the presence and absence of EGCG.

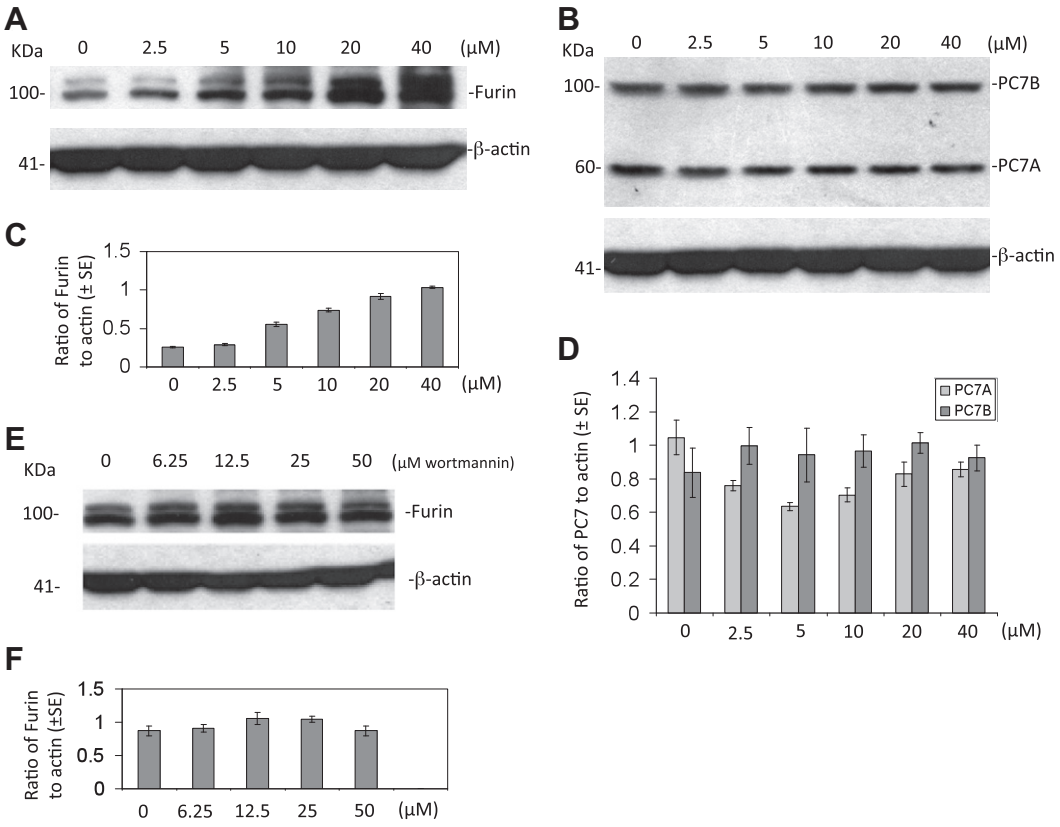

Fig. 5. EGCG enhances ADAM10 activating enzyme furin independent of PI3K activation in SweAPP N2a cells. (A and B) Expression of furin and PC7 was analyzed in lysates from SweAPP N2a cells treated with EGCG at concentrations indicated for 4 h by western blot. (C and D) Densitometric analysis reveals the band density ratio of furin or PC7 isoforms to β-actin (internal reference control). One-way ANOVA followed by *post hoc* comparison revealed significant differences ($P < 0.01$, $n = 3$, data presented as ±S.E.M.) when comparing each concentration of EGCG and respective furin to actin ratio either to control (PBS) or vs. other EGCG dose. Interestingly EGCG treatments did not significantly affect PC7 isoforms expression (B and D). (E) Expression of furin was analyzed in lysates from SweAPP N2a cells treated with EGCG (20 μM) in the presence of PI3K inhibitor (wortmannin) at concentrations indicated for 4 h by western blot. (F) Densitometric analysis reveals the band density ratio of furin to β-actin (internal reference control). One-way ANOVA followed by *post hoc* comparison revealed no significant differences ($n = 3$, data presented as ±SEM) when comparing each concentration of PI3K inhibitor and respective furin to β-actin ratio.

4. Discussion

Therapeutic modalities that oppose cleavage of APP into Aβ peptides and attenuate resultant cerebral amyloidosis have become a primary focus in the last decade. The main targets have been β- and γ-secretases, the two proteases that cleave APP at the N- and C-terminus of the Aβ peptide and are thus directly responsible for Aβ peptide generation. Although mechanistically promising, early clinical studies aimed at treating amyloid-associated neurodegenerative disease by modulating these proteases have been disappointing, likely because adequate doses for treatment are limited by clinical toxicity [52]. A different strategy, namely the activation of α-secretase, has only recently begun to be evaluated for its therapeutic potential despite the fact that it cleaves within the Aβ peptide domain and thus precludes Aβ peptide generation [2–4,7].

Although estrogen replacement therapy remains controversial due largely to adverse effects reported in clinical studies looking at its use in postmenopausal women, the development of selective estrogen modulators for AD continues to be pursued vigorously [14,22]. EGCG, a known modulator of APP processing with function at the ER, is believed to be responsible for the health benefits associated with the consumption of green tea, and has been shown by pharmacokinetic and safety studies to be generally well tolerated [53]. Our laboratory has previously shown that EGCG can increase non-amyloidogenic processing of APP through promotion of the α-secretase ADAM10, which consequently reduced Aβ deposition and improved cognition in AD mice [36,37]. In the present study, we further characterize the mechanisms responsible for EGCG's stimulation of ADAM10 in SweAPP N2a cells by elucidating the involvement of key effectors including ERα, PI3K and Akt (Figs. 1–5). In addition, we corroborate a role for furin in ADAM10 activation and present evidence suggesting that EGCG upregulates furin by mechanisms independent from the ERα/PI3K/ADAM10 pathway proposed here (Fig. 5 and Supplementary Fig. 1).

As suggested by our prior studies, SweAPP N2a cells treated with EGCG displayed a dramatic increase in the mature active form of ADAM10, associated with enhanced metabolites indicative of non-amyloidogenic APP processing (Fig. 1). When these cells were treated concurrently with the selective ERα antagonist MPP and EGCG, dose-dependent reductions in ADAM10 maturation were observed. The ERα agonist PPT appeared to increase ADAM10 maturation alone, but also possibly compete for ER binding in the presence of EGCG; results which further support the involvement of ERα in EGCG's promotion of non-amyloidogenic processing of APP (Fig. 1 and Supplementary Fig. 1). As suggested by other groups, this effect may represent direct activation of membrane associated ERs [42]. Consistent with our and others' findings implicating regulated signal transduction mechanisms mediating EGCG's non-amyloidogenic properties, EGCG failed to show a significant ability to enhance activation of ADAM10 in broken cell preparations (Fig. 2).

Our finding that EGCG may act through the ER is consistent with previous oncologic findings in which EGCG was capable of

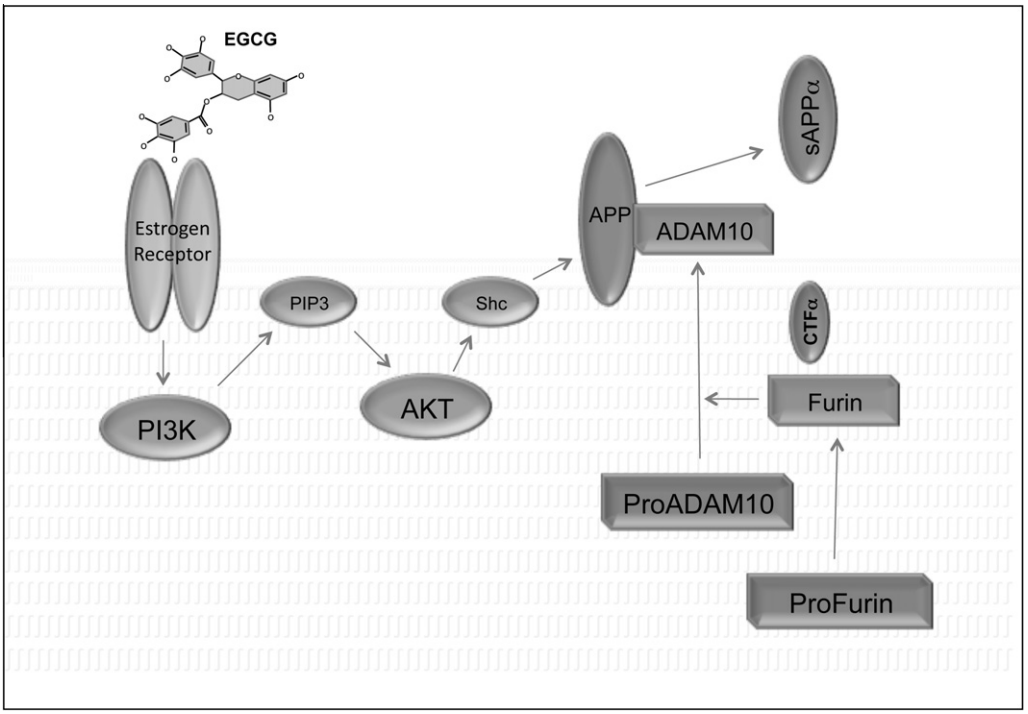

Fig. 6. Working model of effects of EGCG on APP processing. In this model, EGCG activates membrane associated estrogen receptors in a ligand dependent, non-genomic, manner setting in motion receptor tyrosine phosphorylation of the p85 regulatory subunit of PI3K. Subsequently PIP_2 is converted to PIP_3, which, in turn, activates Akt to negatively regulate GSK3 and numerous other downstream effectors required for cellular growth and survival. In our model, Akt may also act directly on APP by phosphorylating C-terminal tyrosine sites or indirectly through the adaptor protein Shc to effect APP phosphorylation [74]. Substrate modifications such as phosphorylation and/or association with adaptor proteins may enhance the binding capacity and substrate-mediated activation of ADAM10 directly [71] or indirectly through autoactivation [51] and enhanced expression of furin, also observed after EGCG treatment.

binding to and downregulating ERα and ERβ [42,54,55]. Taken together, these data suggest a mechanistic clue as to why gender differences and estrogen depletion have been described in AD and other APP related disorders [56–59]; including multiple studies which support an association between certain polymorphisms of the ESR1 gene and the risk of developing AD [60–63].

Although several signal transduction pathways have been implicated in ER and EGCG-mediated pathways, we decided to focus on the PI3K second messenger system as this system canonically involves modulation of GSK-3; a molecule considered to play a key role in AD via the regulation of presenilins and tau [64–66], and ERα has been shown to interact with PI3K/Akt/GSK3 signaling in neuronal cells [44]. However, controversy exists over whether ER-mediated neuroprotection is dependent on PI3K/Akt activation, but not MAPK/ERK signaling [67,68] or whether the coordinated activity of Akt and ERK is responsible for signaling via the ER [69,70]. Whereas the involvement of PI3K signaling subsequent to activation of ERs is well known, comparatively fewer studies have shown EGCG's capacity to activate PI3K in various cell type [35,47]. We find here that SweAPP N2a cells treated with the PI3K inhibitors and EGCG display lower concentrations of sAPPα in media, less activated ADAM10, and less phospho-Akt. Accordingly, PI3K-phospho-p85 was upregulated after EGCG treatment (Fig. 3D); implicating involvement of PI3K in EGCG-mediated ADAM10 activation. Despite these findings, involvement of ERα/MAPK/ERK signaling in EGCG-mediated ADAM10 activation cannot be ruled out.

ADAM10 is known to be regulated by proprotein convertases, especially PC7 and furin [50]. Subsequent experiments aimed at determining whether the EGCG-mediated ERα-PI3K/Akt/ADAM10 pathway involved PC7 or furin upregulation yielded surprising results. As seen in Fig. 5, EGCG enhanced ADAM10 activation was associated with dramatic elevations in furin protein, however this furin upregulation was neither affected by PI3K inhibition nor altered by ERα modulation (Supplementary Fig. 1). Collectively it appears that EGCG enhances ADAM10 activation in both a furin-independent manner via the ERα/PI3K pathway and in a divergent furin dependent manner. Furthermore, PI3K-independent, ERα/MAPK/ERK signaling could also be responsible for furin upregulation in the context of ECGC treatment in these cells. Taken with other reports, this suggests that tight cellular control of this α-secretase is maintained by multiple independently regulated mechanisms, and speaks to the importance of ADAM10's role in cellular function [71,72] (reviewed by Thomas [73]).

In summary, a possible model for activation of ADAM10 by EGCG in SweAPP N2a cells is represented in Fig. 6 (see figure legend for details). Altogether the events depicted, individually or synergistically, lead to enhanced ADAM10 maturation and promotion of non-amyloidogenic processing of APP following EGCG treatment.

Our findings support a role for therapeutic selective ERα modulation in the attenuation or prevention of toxic oligomeric Aβ species formation in AD and related disorders. In addition, our data provide a basic mechanistic rationale for previous clinical findings revealing an increased risk of AD in the context of age-dependent estrogen depletion in women. Further exploration of EGCG's effects on estrogen modulation, activation of ADAM10, and promotion of non-amyloidogenic APP processing is warranted to support the use of this compound as a safe alternative to estrogen replacement therapy in the prevention and treatment of AD.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.09.022.

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