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Resveratrol Promotes Clearance of Alzheimer's Disease Amyloid-β Peptides*

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Philippe Marambaud[1], **Haitian Zhao, and Peter Davies**

From the Litwin-Zucker Research Center for the Study of Alzheimer's Disease and Memory Disorders, North Shore-Long Island Jewish Institute for Medical Research, Manhasset, New York 11030

Several epidemiological studies indicate that moderate consumption of wine is associated with a lower incidence of Alzheimer's disease. Wine is enriched in antioxidant compounds with potential neuroprotective activities. However, the exact molecular mechanisms involved in the beneficial effects of wine intake on the neurodegenerative process in Alzheimer's disease brain remain to be clearly defined. Here we show that resveratrol (*trans*-3,4′,5-trihydroxystilbene), a naturally occurring polyphenol mainly found in grapes and red wine, markedly lowers the levels of secreted and intracellular amyloid-β (Aβ) peptides produced from different cell lines. Resveratrol does not inhibit Aβ production, because it has no effect on the Aβ-producing enzymes β- and γ-secretases, but promotes instead intracellular degradation of Aβ via a mechanism that involves the proteasome. Indeed, the resveratrol-induced decrease of Aβ could be prevented by several selective proteasome inhibitors and by siRNA-directed silencing of the proteasome subunit β5. These findings demonstrate a proteasome-dependent anti-amyloidogenic activity of resveratrol and suggest that this natural compound has a therapeutic potential in Alzheimer's disease.

Alzheimer's disease (AD)[2] is a progressive neurodegenerative disorder leading to the most common form of dementia. Compelling evidence supports the central role of Aβ in the pathogenesis of the disease (1). Aβ is a core component of the senile plaque, a classical lesion found in the neocortex and hippocampus of AD brains, and excessive production of the highly insoluble 42-amino acid-long Aβ42 peptide is almost invariably observed in the presence of mutations in the three genes linked to early onset autosomal dominant familial forms of AD (2).

In the amyloidogenic pathway, the amyloid-β precursor protein (APP) is cleaved by the aspartic protease β-secretase/BACE1 to yield the membrane-anchored C-terminal fragments C99 and C89. C99 is then endoproteolyzed by the γ-secretase proteolytic complex to produce various Aβ peptides. The major cleavage takes place after Val-40 producing Aβ40. In an alternative nonamyloidogenic pathway, APP is endoproteolyzed within the Aβ region by α-secretase to generate the C-terminal fragment C83 and the soluble N-terminal fragment secreted APPα. Finally, a γ-secretase-mediated ε-cleavage of APP allows the intracellular release of the transcriptionally active APP intracellular domain (AID (3) or AICD) (4–6).

Epidemiological studies have shown that moderate wine intake reduces the risk of developing AD (7–10). Resveratrol, a polyphenol that occurs in abundance in grapes and red wine, is suspected to afford antioxidant and neuroprotective properties and therefore to contribute to the beneficial effect of wine consumption on the neurodegenerative process (11–13). Here we report that resveratrol has a potent anti-amyloidogenic activity by reducing the levels of Aβ produced from different cell lines expressing wild type or Swedish mutant APP_{695}. We show that resveratrol acts by promoting the intracellular degradation of Aβ by a mechanism that implicates the proteasome.

EXPERIMENTAL PROCEDURES

Materials and Antibodies—Quercetin, catechin, resveratrol, piceatannol, phosphoramidon, thiorphan, insulin, *N*-succinyl-LLVY-7-amido-4-methylcoumarin (Suc-LLVY-AMC), and Suc-AAF-AMC were obtained from Sigma. Trimethoxy-resveratrol and TMS (*trans*-2,3′,4,5′-tetramethoxystilbene) were from Cayman Chemical. L-685,458, lactacystin, Z-GPFL-CHO, YU101, and *N*-acetyl-LL-norleucinal-CHO (ALLN) were from Calbiochem. Purified human 20 S proteasome was from Biomol. Anti-Aβ-(1–17) (6E10) and anti-Aβ-(17–24) (4G8, pure and biotinylated) antibodies were from Signet. Anti-APP-(66–81) (22C11) antibody was from Chemicon, and anti-APP C-terminal domain (R1) antibody was provided by Dr. P. D. Mehta, Institute for Basic Research in Developmental Disabilities, Staten Island, NY. Polyclonal antibodies specific for Aβ40 (FCA3340) or Aβ42 (FCA3542) (14) were obtained from Dr. F. Checler, IPMC-Centre National de la Recherche Scientifique, Valbonne, France. Anti-N-cadherin (C32) and anti-β-catenin antibodies were from BD Transduction Laboratories. Anti-20 S proteasome subunit β5 antibody was from ABR Affinity BioReagents, and the polyclonal antibody directed against the subunits α5, α7, β1, β5, β5i, and β7 of the 20 S proteasome was from Biomol. Anti-β-tubulin antibody was from Santa Cruz Biotechnology.

Cell Lines, Transfections, and Drug Treatments—HEK293 cells stably transfected with human APP_{695} were provided by Dr. L. D'Adamio, Albert Einstein College of Medicine, Bronx, NY. N2a cells were stably transfected with wild type or Swedish mutant human APP_{695} cDNAs (obtained from Dr. N. K. Robakis, Mount Sinai School of Medicine, New York, NY). APP_{695}-HEK293 transfectants were grown in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum, penicillin and streptomycin, and 5 μg/ml puromycin. APP_{695}-N2a cells were maintained in 1:1 Dulbecco's modified Eagle's medium/Opti-MEM supplemented with 5% fetal bovine serum, penicillin and streptomycin, and 0.2 mg/ml G418. For drug treatments, cells were treated at confluence for the indicated concentrations and incubation times. Medium was then

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[1] To whom correspondence should be addressed: The Litwin-Zucker Research Center for the Study of Alzheimer's Disease and Memory Disorders, North Shore-Long Island Jewish Institute for Medical Research, 350 Community Dr., Manhasset, NY 11030. Tel.: 516-562-3492; E-mail: pmaramba@aecom.yu.edu.

[2] The abbreviations used are: AD, Alzheimer's disease; Suc, succinyl; TMS, *trans*-2,3′,4,5′-tetramethoxystilbene; ALLN, *N*-acetyl-LL-norleucinal-CHO; ELISA, enzyme-linked immunosorbent assay; ECE, endothelin-converting enzyme; IDE, insulin-degrading enzyme; Aβ, amyloid-β; APP, amyloid-β precursor protein; AMC, amido-4-methylcoumarin; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; WB, Western blotting; IP, immunoprecipitation; NEP, neprilysin; MOPS, 4-morpholinepropanesulfonic acid; ER, endoplasmic reticulum; siRNA, small interfering RNA; TBS, Tris-buffered saline; sAβ, secreted amyloid-β; Z, benzyloxycarbonyl.

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changed, and treatments were continued for another 2 h to allow Aβ secretion. For siRNA-directed silencing, 200 pmol of purified siRNA directed against the proteasome subunit β5 (SMARTpool, Dharmacon) were transfected with 10 μl of Lipofectamine 2000 (Invitrogen) in APP_{695}-HEK293 cells plated in 35-mm dishes. At 48 h post-transfection, cells were incubated in the absence or presence of 40 μM resveratrol for another 24 h. Cells and conditioned medium were harvested and analyzed by Western blotting (WB) and by proteasome activity assays as described below.

Western Blotting—Cells were washed with phosphate-buffered saline and solubilized in ice-cold HEPES buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, 1× Complete protease inhibitor mixture, Roche Applied Science) containing 1% SDS. Ten micrograms of extracts were analyzed by SDS-PAGE. For total sAβ WB, conditioned medium was subjected to 0.2-μm filtration. Twenty microliters of medium were then electrophoresed on 16.5% Tris-Tricine gels and transferred onto 0.2-μm nitrocellulose membranes. Membranes were microwaved for 5 min in phosphate-buffered saline, blocked in 5% fat-free milk in TBS, and incubated with 6E10 (1:1000 in Pierce SuperBlock) overnight at 4 °C. A standard ECL detection procedure was then used.

Aβ Immunoprecipitations (IPs)—Cells were solubilized in ice-cold RIPA buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1× Complete). Five hundred micrograms of cell extracts (for total intracellular Aβ IP) or 1 ml of the corresponding conditioned medium diluted in 4× RIPA buffer (for sAβ40 and sAβ42 IPs) were precleared with protein A- or protein G-Sepharose (Amersham Biosciences) for 2 h at 4 °C. Supernatants were then incubated overnight at 4 °C with 3 μl of antibodies 4G8 (total intracellular Aβ IP), FCA3340 (sAβ40 IP), or FCA3542 (sAβ42 IP). Supernatants were then treated for 2 h at 4 °C with protein A-Sepharose (polyclonal antibodies) or with protein G-Sepharose (monoclonal antibodies). IPs were washed with ice-cold RIPA buffer and analyzed by WB using the 6E10 antibody as described above.

Aβ Enzyme-linked Immunosorbent Assay (ELISA)—6E10 (capture antibody) was coated at 2 μg/ml in coating buffer (2.27 g/liter K_2HPO_4, 3.48 g/liter KH_2PO_4, pH 7.2, 8 g/liter NaCl, 0.372 g/liter EDTA, 0.1 g/liter NaN_3) into 96-well immunoassay plates for 24 h at 4 °C. The plates were washed with 0.05% Tween 20 in TBS (TTBS) and blocked with Pierce TBS starting block buffer for 1 h at room temperature. The samples (conditioned medium or Aβ1–40 standards) and biotinylated 4G8 (reporter antibody, at 0.5 μg/ml in 20% Pierce SuperBlock) were then added to the plates and incubated at room temperature for 2 h. Following washing with TTBS, streptavidin-horseradish peroxidase (Southern Biotech, at 0.25 μg/ml in 20% SuperBlock) was added to the wells for 1 h at room temperature. The fluorogenic substrate Amplex Ultra Red (Molecular Probes) was added to the plates and incubated for 15 min. Reaction products were quantified using a Tecan Genios Pro plate reader at 535 nm excitation and 590 nm emission.

Enzymatic Activity Assays—For neprilysin (NEP) activity assays, intact cells were incubated at 37 °C for 2 h in Opti-MEM containing 50 μM Suc-AAF-AMC in the absence or presence of 20 μM thiorphan. Cells were then homogenized, and protein concentrations were determined using a Bradford assay (Bio-Rad). Chymotrypsin-like activity of the endogenous proteasome was determined by solubilizing the cells in activity assay buffer (25 mM HEPES, pH 7.5, 0.5 mM EDTA, 0.05% Nonidet P-40, and 0.001% SDS). Cell extracts (40 μg) were incubated at 37 °C for 2 h in 100 μl of activity assay buffer containing 50 μM Suc-LLVY-AMC in the absence or presence of 10 μM of ALLN. For purified proteasome activity assays, 12.5 μg/ml purified human 20 S proteasome were incubated at 37 °C for 2 h in 100 μl of activity assay buffer contain-

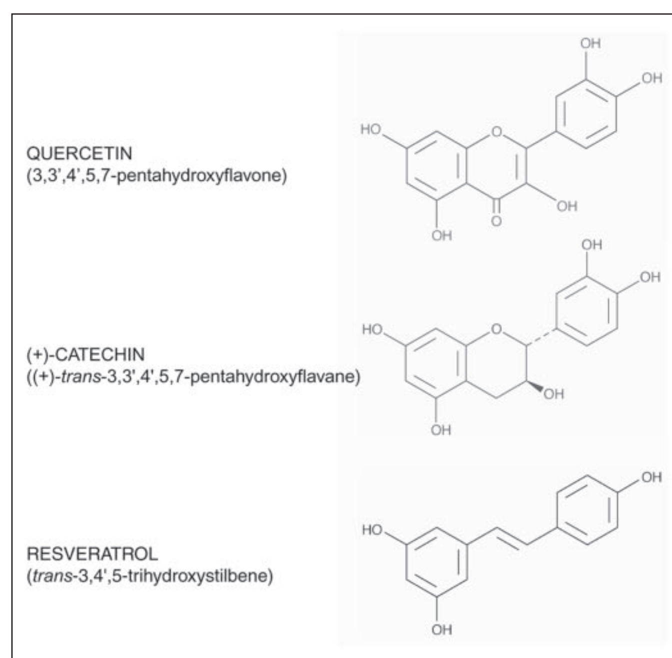

FIGURE 1. **Structure of quercetin, catechin, and resveratrol.**

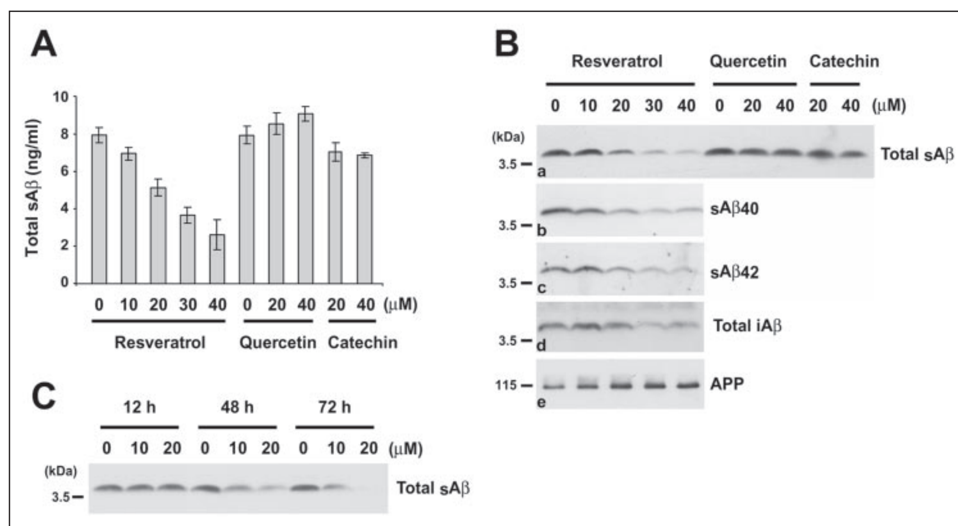

FIGURE 2. **Effect of resveratrol, quercetin, and catechin on Aβ levels in APP_{695}-HEK293 cells.** *A* and *B*, cells were treated for 24 h with the indicated concentrations of polyphenols (dissolved in Me_2SO). Medium was changed, and drug treatments were continued for another 2 h to allow Aβ secretion. Total sAβ was analyzed by ELISA (*A*) and WB (*B*, *panel a*). *B*, sAβ40 (*b*), sAβ42 (*c*), and total intracellular Aβ (*panel d*, *total iAβ*) were analyzed by IP and WB (see "Experimental Procedures"). Full-length APP (*B*, *panel e*) was analyzed by WB. *C*, cells were treated for different periods of time with the indicated concentrations of resveratrol. Total sAβ was then analyzed as in *B*. The final concentration of Me_2SO was adjusted to 0.04% (v/v) in all conditions. Histogram shows the mean ± S.D. of 3–4 independent experiments. The Western blots shown are representative of at least three independent experiments.

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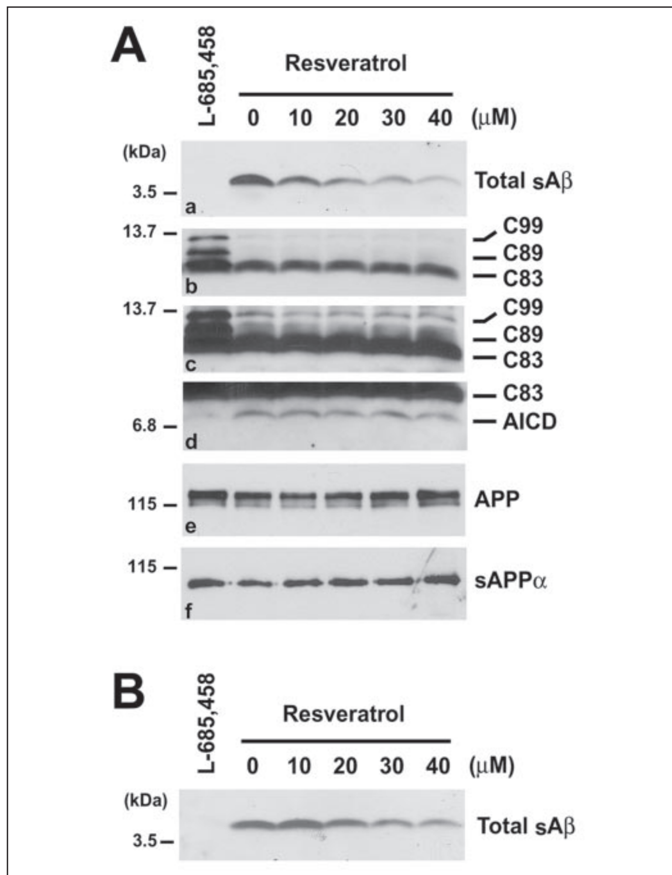

FIGURE 3. **Effect of resveratrol on APP processing in N2a cells expressing wild type or Swedish APP_{695}.** *A*, wild type APP_{695}-N2a cells were treated for 24 h with increasing concentrations of resveratrol or with 1 μM γ-secretase inhibitor, L-685,458 (*first lane*). Medium was changed, and drug treatments were continued for another 2 h to allow Aβ secretion. Total sAβ levels were then analyzed by WB (*panel a*). APP C-terminal fragments, C99, C89, C83 (*panel b*, short film exposure; *panel c*, long film exposure) and APP intracellular domain (*AICD*, *panel d*) were analyzed by WB using R1 antibody. Full-length APP (*panel e*) and secreted APPα (*sAPPα*, *panel f*) were probed with antibodies 22C11 and 6E10, respectively. *B*, Swedish APP_{695}-N2a cells were treated with resveratrol or L-685,458 as described in *A*. Total sAβ was then analyzed as above. The Western blots shown are representative of at least three independent experiments.

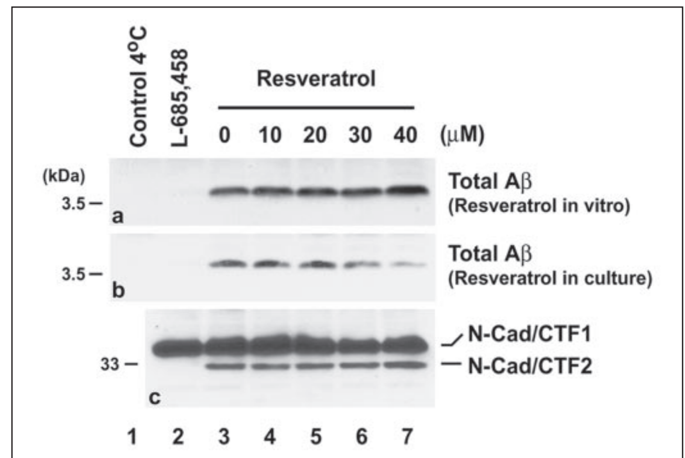

FIGURE 4. **Resveratrol does not affect γ-secretase activity *in vitro*.** *Panel a*, membrane preparations from APP_{695}-HEK293 cells (see "Experimental Procedures") were incubated *in vitro* at 4 °C (*lane 1*) or 37 °C (*lanes 2–7*) for 4 h in the absence or presence of the indicated drugs. Total Aβ produced *in vitro* was analyzed by WB with 6E10 antibody. *Panels b* and *c*, membranes from APP_{695}-HEK293 cells treated in culture for 24 h in the absence or presence of the indicated drugs were incubated as described above. Total Aβ and N-cadherin-derived C-terminal fragments (*N-Cad/CTF1* and *N-Cad/CTF2*) were analyzed by WB with antibodies 6E10 (*panel b*) and C32 (*panel c*), respectively.

ing 50 μM Suc-LLVY-AMC. Release of AMC was measured by fluorescence spectrophotometry using wavelengths of 340 nm excitation and 535 nm emission (Tecan Genios Pro). Enzymatic activities were expressed as nmol AMC/min/mg protein.

In Vitro γ-Secretase Assays—In vitro assays were performed as described previously (15). Briefly, cells were resuspended in 0.5 ml/35-mm dish of hypotonic buffer (10 mM MOPS, pH 7.0, 10 mM KCl) and homogenized on ice. A postnuclear supernatant was prepared by centrifugation at 1000 × *g* for 15 min at 4 °C. Crude membranes were isolated from the postnuclear supernatant by centrifugation at 16,000 × *g* for 40 min at 4 °C. The membranes were then resuspended in 25 μl of assay buffer (150 mM sodium citrate, pH 6.4, 1× Complete) and incubated at 37 °C for 4 h in the absence or presence of the indicated drugs. Samples were then analyzed by WB.

RESULTS

Because evidence is increasing that moderate wine intake reduces the risk of developing AD (7–10), we sought to determine whether three powerful antioxidant polyphenols found in red wine, resveratrol (*trans*-3,4′,5-trihydroxystilbene, see Fig. 1), quercetin (3,3′,4′,5,7-pentahydroxyflavone), and catechin ((+)-*trans*-3,3′,4′,5,7-pentahydroxyflavane), modulate Aβ levels. To this end, we treated APP_{695}-transfected HEK293 cells with increasing concentrations of the different polyphenols and analyzed Aβ levels by ELISA and WB. Total secreted Aβ (Fig. 2, *A* and *B*, *panel a*), including secreted Aβ40 (Fig. 2*B*, *panel b*) and Aβ42 (Fig. 2*B*, *panel c*), were markedly reduced by 20–40 μM resveratrol after 24 h of incubation, whereas quercetin and catechin were apparently ineffective at these concentrations (Fig. 2, *A* and *B*, *panel a*). At the same concentration range, resveratrol also reduced total intracellular Aβ (Fig. 2*B*, *panel d*). Because resveratrol treatment did not lead to intracellular accumulation of Aβ (Fig. 2*B*, *panel d*), we concluded that Aβ secretion was not impaired. To determine whether the effect of resveratrol is time-dependent, APP_{695}-HEK293 cells were then treated for different periods of time with 10 or 20 μM polyphenol. Fig. 2*C* shows that, although resveratrol did not affect Aβ levels after 12 h of incubation, its inhibitory effect on Aβ levels is gradually strengthened after longer incubation periods of 48 and 72 h.

To exclude a cell line-specific effect, we also treated APP_{695}-transfected mouse neuroblastoma N2a cells with resveratrol. This showed that resveratrol inhibited total secreted Aβ at the same concentration range in another cell line (Fig. 3*A*, *panel a*). Secreted Aβ produced by N2a cells overexpressing APP_{695} bearing the familial AD Swedish mutation, was also reduced by similar treatments (Fig. 3*B*). To determine whether resveratrol affects APP metabolism, we then monitored by WB the levels of APP holoprotein and proteolytic fragments. At the same concentrations, resveratrol neither affected full-length APP levels (Fig. 2*B*, *panel e*, and Fig. 3*A*, *panel e*) nor reduced secreted APPα immunoreactivity (Fig. 3*A*, *panel f*). In addition, resveratrol did not affect the steady-state levels of APP C-terminal fragments C99, C89, and C83 (Fig. 3*A*, *panels b* and *c*) and APP intracellular domain (*AICD*, Fig. 3*A*, *panel d*). Together these data indicate that resveratrol had no effect on the α-, β-, or γ-secretase-mediated cleavages of APP or on the stability of APP or its C-terminal fragments. Therefore, resveratrol did not affect APP metabolism and Aβ production.

To confirm that resveratrol did not inhibit β- or γ-secretase directly, resveratrol was used in a cell-free assay designed to produce Aβ *in vitro*. In this *in vitro* assay, membrane preparations isolated from APP_{695}-HEK293 cells were incubated at 37 °C for 4 h in the absence or presence of increasing concentrations of resveratrol. Production of Aβ was not affected by the presence of resveratrol *in vitro* (Fig. 4*a*). However, the

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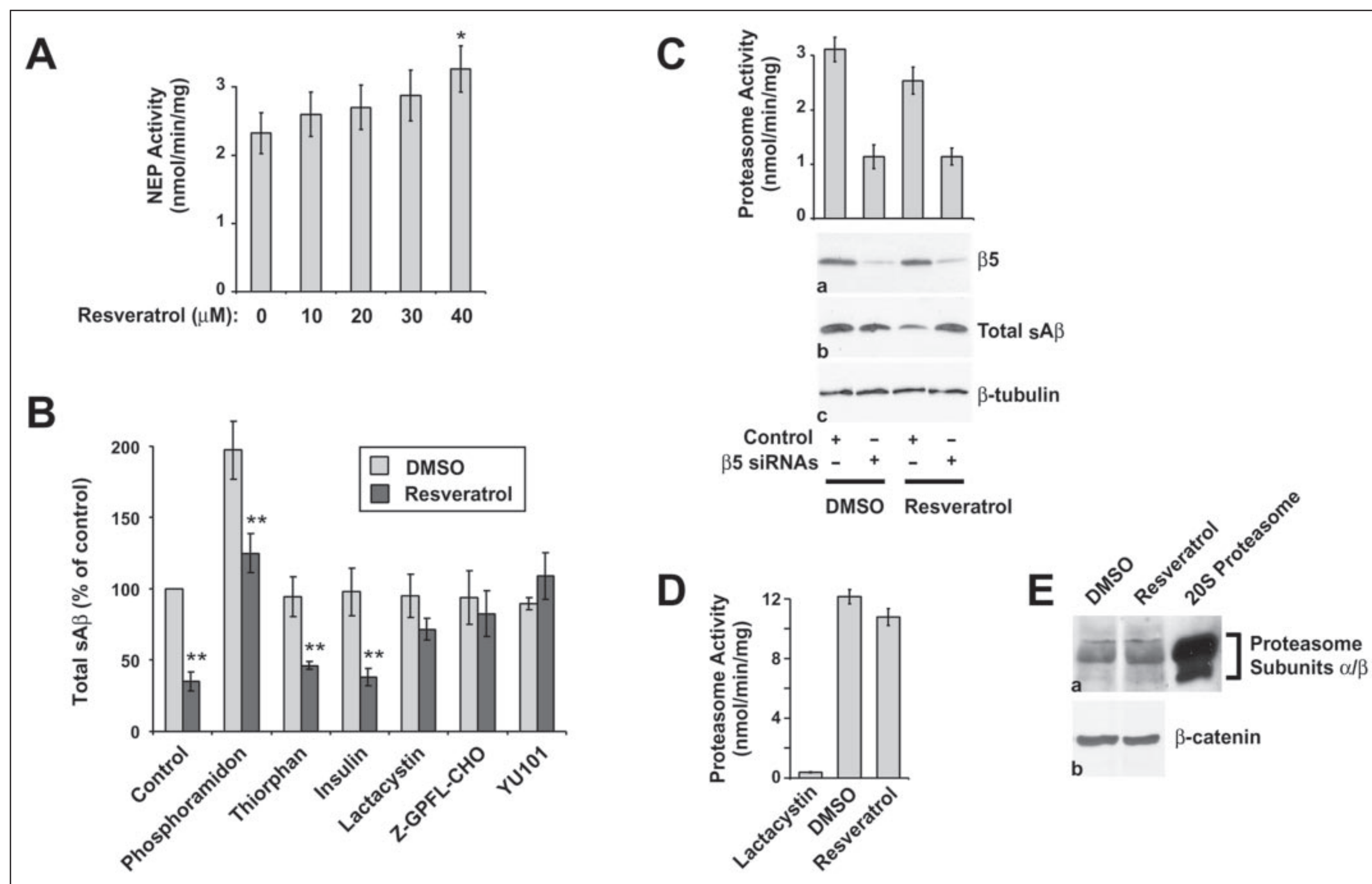

FIGURE 5. **Proteasome inhibition prevents the resveratrol-mediated decrease of Aβ.** *A*, APP_{695}-HEK293 cells were incubated for 24 h with increasing concentrations of resveratrol. Thiorphan-sensitive endoproteolytic activity (*NEP Activity*) was then determined on intact cells as described under "Experimental Procedures." *B*, APP_{695}-HEK293 cells were treated for 24 h with resveratrol (40 μM) or its vehicle (dimethyl sulfoxide, *DMSO*) in the absence (*Control*) or presence of phosphoramidon (50 μM), thiorphan (20 μM), insulin (10 μM), lactacystin (2 μM), Z-GPFL-CHO (25 μM), or YU101 (1 μM). Total sAβ was then analyzed by ELISA as described in Fig. 2*A*. *C*, APP_{695}-HEK293 cells transfected with β5 siRNAs or buffer only (*Control*) were treated for 24 h with resveratrol (40 μM) or its vehicle (dimethyl sulfoxide, *DMSO*). Cells were then harvested and analyzed by WB with anti-β5 antibody (*panel a*) and with anti-β-tubulin antibody (*panel c*). Total sAβ (*panel b*) was analyzed by WB as described in Fig. 2*B*. ALLN-sensitive endoproteolytic activity (proteasome activity) was determined as described under "Experimental Procedures." *D*, purified human 20 S proteasome was incubated for 30 min with lactacystin (20 μM), dimethyl sulfoxide, or resveratrol (40 μM). Proteasome activity was then monitored as in *C*. *E*, APP_{695}-HEK293 cells were treated for 24 h with dimethyl sulfoxide or resveratrol (40 μM). Cells were then harvested and analyzed by WB with anti-20 S proteasome subunits α/β antibody (*panel a*) and with anti-β-catenin antibody (*panel b*). Five hundred nanograms of purified human 20 S proteasome were analyzed by WB (*panel a*, *last lane*). Values are the mean ± S.D. of 3–5 independent experiments. *, $p < 0.05$; **, $p < 0.01$ (Student's *t* test).

levels of Aβ produced *in vitro* by membranes isolated from cells treated in culture with the polyphenol were significantly reduced (Fig. 4*b*), indicating that resveratrol promoted Aβ reduction without directly affecting β- and γ-secretases.

γ-Secretase targets several other type I proteins, including the cell-cell adhesion receptors E- and N-cadherins (15, 16). Cleavage of N-cadherin by γ-secretase produces the transcriptionally active intracellular fragment N-Cad/CTF2 from the proteolytic cleavage of the intermediate C-terminal fragment N-Cad/CTF1 (15). Using a similar cell-free assay we determined that production of N-Cad/CTF2 was not affected by resveratrol treatment (Fig. 4*c*), confirming the absence of inhibitory effect of resveratrol on γ-secretase activity. Together with the observation that resveratrol did not affect the levels of APP holoprotein and its C-terminal proteolytic fragments, these data indicate that resveratrol did not target an Aβ-producing activity but rather promoted Aβ clearance.

Based on these observations, we aimed to determine whether resveratrol treatment promotes Aβ degradation. Aβ peptides are degraded *in vivo* by at least four metalloendopeptidases, NEP, endothelin-converting enzyme-1 and -2 (ECE-1 and -2), and insulin-degrading enzyme (IDE) (17). Recent evidence indicates that long term treatment with resveratrol promotes NEP activity in SK-N-SH cells (18). Using thiorphan-sensitive enzymatic assays on intact HEK293 cells, we confirmed that NEP activity was significantly increased upon resveratrol treatment (Fig. 5*A*). However, inhibition of NEP with phosphoramidon or thiorphan in APP_{695}-HEK293 cells could not prevent the decrease of Aβ levels triggered by resveratrol (Fig. 5*B*). Because phosphoramidon also inhibits ECE-1 and -2, we concluded that these enzymatic activities were also not involved in the resveratrol-mediated decrease of Aβ. Further, pretreatment with insulin, which acts as a competitive inhibitor of IDE, did not rescue Aβ levels during resveratrol treatment (Fig. 5*B*). Thus, resveratrol did not promote Aβ degradation by NEP, ECE-1 and -2, or IDE in HEK293 cells.

Converging evidence indicates that resveratrol promotes the proteasomal degradation of a specific subset of proteins, including cyclin D1 (19), the estrogen receptor-α (20), or the hypoxia-inducible factor-1α (21). Because the proteasome has been shown to modulate Aβ levels (22), we asked whether proteasome inhibition rescues Aβ levels upon resveratrol treatment. Fig. 5*B* shows that treatments with the selective proteasome inhibitors, lactacystin, Z-GPFL-CHO, or YU101, significantly prevented the resveratrol-induced decrease of Aβ. To confirm the involvement of the proteasome in the anti-amyloidogenic effect of resveratrol, we sought to down-regulate proteasome activity by siRNA-directed silencing. The proteasome is a multicatalytic protease complex

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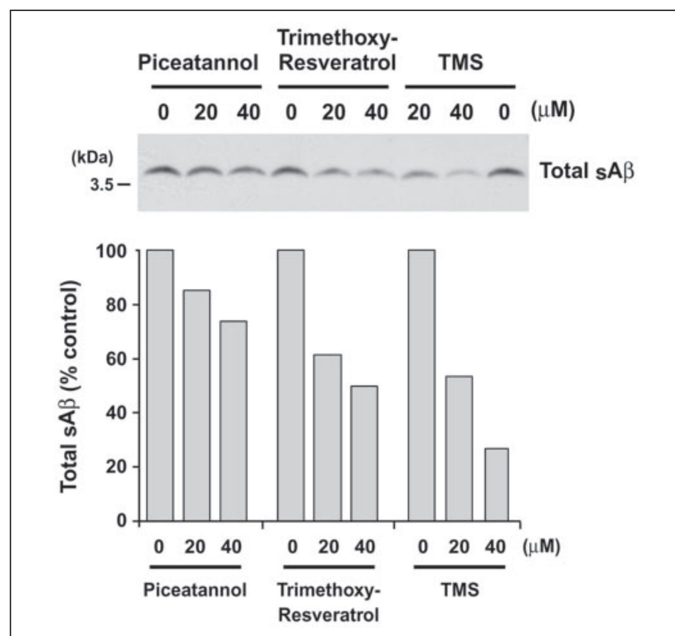

FIGURE 6. **Effect of resveratrol analogues on Aβ levels.** APP_{695}-HEK293 cells were treated for 24 h with the indicated concentrations of resveratrol analogues (dissolved in Me_2SO). Total sAβ was analyzed as in Fig. 2*B*. The final concentration of Me_2SO was adjusted to 0.04% (v/v) in all conditions. The histogram shows the relative amounts of total sAβ detected by WB in the *upper panel*.

formed by different subunits encoded by several genes (23). Because YU101 is highly selective for the chymotrypsin-like activity of the proteasome (24) and because the subunit β5 is critical for this proteolytic activity (23), we asked whether siRNA-directed silencing of the proteasome subunit β5 prevents the resveratrol-induced decrease of Aβ. Transfection of siRNAs directed against the subunit β5 strongly decreased β5 protein expression (Fig. 5*C*, *panel a*) and inhibited more than 60% of the chymotrypsin-like activity of the proteasome (Fig. 5*C*). Under these conditions, we observed a strong inhibition of the resveratrol-induced Aβ decrease (Fig. 5*C*, *panel b*).

We then investigated whether resveratrol directly stimulates proteasome activity. In Fig. 5*D*, the chymotrypsin-like activity of purified proteasome was monitored upon *in vitro* incubation with resveratrol. The same activity was also assessed from endogenous proteasome in cell extracts after treatment with the polyphenol in cell cultures (Fig. 5*C*). No significant effect of resveratrol on the chymotrypsin-like activity of purified or endogenous proteasome was observed (histograms in Fig. 5, *D* and *C*, respectively). Because resveratrol effectively reduced Aβ levels after 24 h of incubation, we also asked whether resveratrol modifies proteasome subunit transcription. We found that resveratrol treatment does not markedly affect the steady-state levels of several proteasome subunits in HEK293 cells (Fig. 5*C*, *panel a*, and Fig. 5*E*, *panel a*). Together these results demonstrate that resveratrol promotes a proteasome-dependent intracellular degradation of Aβ via a mechanism that does not increase total proteasome activity. Consistent with this conclusion, levels of β-catenin, a cytosolic protein degraded by the ubiquitin proteasome system (25), are not affected by resveratrol in HEK293 cells (Fig. 5*E*, *panel b*).

We then investigated the anti-amyloidogenic effect of several resveratrol analogues. Three analogues were tested: piceatannol (*trans*-3,3′,4,5′-tetrahydroxystilbene), which contains an additional hydroxyl group at C-3, and two methoxy analogues, trimethoxy-resveratrol (*trans*-3,4′,5-trimethoxystilbene) and TMS. Interestingly, the three analogues were able to decrease Aβ levels (Fig. 6). However, compared with resveratrol (Fig. 2*A*), piceatannol and trimethoxy-resveratrol were less potent, whereas TMS had a very comparable potency in reducing the amounts of Aβ (Fig. 6).

DISCUSSION

Our data show that resveratrol strongly reduces Aβ produced by different cell lines expressing wild type or Swedish mutant APP_{695}. Resveratrol acts by promoting the intracellular degradation of the amyloid peptide by a mechanism that implicates the proteasome. Pharmacological studies show that none of the previously reported Aβ-degrading metalloendopeptidases, NEP, ECE-1 and -2, or IDE, are involved in this clearance. Finally, we demonstrate the anti-amyloidogenic activity of two methoxy analogues of resveratrol, trimethoxy-resveratrol and TMS, suggesting that chemical modifications of resveratrol can be done in the context of improving its potency, stability, and bioavailability and therefore its therapeutic use.

Additional studies will be needed to fully elucidate the role of the proteasome in this mechanism of intracellular clearance of Aβ. A number of possible functions of the proteasome in the regulation of Aβ metabolism have been ascribed to the multicatalytic complex (22). The proteasome targets for degradation C99 and three core components of the γ-secretase complex, presenilins, APH-1, and Pen-2 (22), suggesting that proteasome activation may decrease Aβ levels by reducing the amounts of C99 available and by altering γ-secretase activity. Our data show no reduction of C99 levels (Fig. 3*A*, *panel c*) or alteration of the γ-secretase-mediated cleavages of APP or N-cadherin upon resveratrol treatment (Figs. 3 and 4), thus excluding the possibility that resveratrol lowers Aβ by promoting the proteasomal degradation of C99 or any γ-secretase components. Recent evidence also suggests that Aβ can be degraded by a proteasome-dependent endoplasmic reticulum (ER)-associated degradation (26). Using cell-free reconstitutions of ER-derived brain microsomes, Schmitz *et al.* (26) show that Aβ can translocate from the ER to the cytosol where it is directly degraded by the proteasome. It is conceivable that resveratrol promotes such a clearance mechanism. However, because ER Aβ represents a small fraction of total Aβ produced and because a small pool of Aβ produced in the ER appears to be controlled by ER-associated degradation, it is unlikely that the severe reduction of Aβ levels observed in the presence of resveratrol is entirely due to an increase of this clearance mechanism. We therefore hypothesize that resveratrol may act indirectly by selectively stimulating the proteasomal degradation of yet to be identified critical regulators of Aβ clearance.

It will be also important to determine what the molecular targets of resveratrol are in the pathway of Aβ clearance. Resveratrol interacts with several proteins, including members of the sirtuin family. Sirtuins are evolutionarily conserved deacetylases with important functions in longevity (27). Resveratrol was found to act as a potent activator of the human sirtuin SIRT1 *in vitro* and of the yeast homologue Sir2 *in vivo*, a mechanism that may extend life span in yeast (28). Moreover, resveratrol and SIRT1 activation have recently been linked to neuroprotective pathways in models of axonal degeneration (29) and of neuronal dysfunctions caused by mutant polyglutamines (30). It would therefore be of interest to determine whether SIRT1 is involved in the resveratrol-induced decrease of Aβ.

Evidence is compelling that a decrease in proteasome activity occurs in AD brains (31, 32). It is unclear, however, whether this decrease in proteasome activity is in parallel with an increase in Aβ levels. It has been proposed that Aβ itself may lead to proteasome inhibition (33), suggesting that high levels of Aβ in AD brain may create a vicious cycle by inhibiting the proteasome and blocking the degradation of critical

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regulators of its own clearance. In this context, our data reveal an important mechanism of selective proteasome activation in the anti-amyloidogenic effect of resveratrol and support the therapeutic potential of this natural polyphenol.

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