Antidiabetic drug metformin (Glucophage^R) increases biogenesis of Alzheimer's amyloid peptides via up-regulating *BACE1* transcription

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Epidemiological, clinical and experimental evidence suggests a link between type 2 diabetes and Alzheimer's disease (AD). Insulin modulates metabolism of β-amyloid precursor protein (APP) in neurons, decreasing the intracellular accumulation of β-amyloid (Aβ) peptides, which are pivotal in AD pathogenesis. The present study investigates whether the widely prescribed insulin-sensitizing drug, metformin (Glucophage^R), affects APP metabolism and Aβ generation in various cell models. We demonstrate that metformin, at doses that lead to activation of the AMP-activated protein kinase (AMPK), significantly increases the generation of both intracellular and extracellular Aβ species. Furthermore, the effect of metformin on Aβ generation is mediated by transcriptional up-regulation of β-secretase (BACE1), which results in an elevated protein level and increased enzymatic activity. Unlike insulin, metformin exerts no effect on Aβ degradation. In addition, we found that glucose deprivation and various tyrphostins, known inhibitors of insulin-like growth factors/insulin receptor tyrosine kinases, do not modulate the effect of metformin on Aβ. Finally, inhibition of AMP-activated protein kinase (AMPK) by the pharmacological inhibitor Compound C largely suppresses metformin's effect on Aβ generation and *BACE1* transcription, suggesting an AMPK-dependent mechanism. Although insulin and metformin display opposing effects on Aβ generation, in combined use, metformin enhances insulin's effect in reducing Aβ levels. Our findings suggest a potentially harmful consequence of this widely prescribed antidiabetic drug when used as a monotherapy in elderly diabetic patients.

Alzheimer's disease (AD) is a devastating neurodegenerative disorder, with aging, genetic, and environmental factors contributing to its development and progression. AD is not only characterized by pathological deposition of Aβ peptides and neurofibrillary tangles but is also associated with microglia-mediated inflammation and dysregulated lipid homeostasis and glucose metabolism. Amyloid peptides are derived from sequential proteolytic cleavages of full-length amyloid precursor protein (APP) by β-secretase (BACE1) and γ-secretase. Full-length APP can undergo alternative processing by α-secretase, releasing a soluble fragment (sAPPα) extracellularly, which precludes Aβ formation. Compelling evidence indicates that Aβ, especially the oligomers, are toxic to neurons; excessive generation and accumulation of Aβ peptides in neurons is believed to initiate the pathological cascade in AD (1–3).

Epidemiological studies strongly suggest that metabolic defects correlate with the functional alterations associated with aging of the brain and with AD pathogenesis (4–11). The vast majority of AD cases are late onset and sporadic in origin with aging being the most profound risk factor. Insulin signaling is known to be involved in the process of brain aging (12–20). Insulin dysfunction/resistance in diabetes mellitus (DM) is not only a common syndrome in the elderly but also considered a risk factor for AD, especially for vascular dementia (21, 22). The link between DM and AD, plus the high prevalence of both diseases in the elderly population, prompted us to search for desirable concomitant pharmacotherapy based on the FDA-approved drugs. Clinical findings indicated that insulin has beneficial effects on cognition in patients with dementia (23, 24). Moreover, clinical trials on the PPARγ agonist rosiglitazone, one of the FDA-approved thiazolidinediones (TZDs) for treating type 2 diabetes, showed improved cognition and memory in patients with mild to moderate AD (25–28). In addition, we have shown that insulin regulates APP processing/trafficking in neuronal cultures, reducing intracellular levels of Aβ (29). In this context, it would be of interest to learn whether another FDA-approved insulin-sensitizing drug, metformin, which likely acts independently of the PPAR pathways, has a similar effect on APP/Aβ metabolism. Metformin (Glucophage^R, 1, 2-dimethylbiguanide hydrochloride; ≈36 million U.S. prescriptions in 2003) (30), is a biguanide that has pleiotropic effects on metabolism, including insulin-sensitization, increased glucose uptake, decreased hepatic glucose synthesis, activation of AMP activated protein kinase (AMPK, an enzyme involved in glucose and fatty acid metabolism), and mitochondria inhibition (31, 32).

Results

Metformin Increases Aβ Generation. To examine the effects of metformin on APP metabolism, we used 2 cellular models including primary cortical neurons and N2a neuroblastoma cells stably expressing human APP. We treated N2a695 cells with metformin and found that metformin increased levels of both extracellular (Fig. 1*A*) and intracellular (data not shown) Aβ40/42 in dose-dependent manners, with the maximum effect (≈3-fold) seen after 24 h at 10 mM. Similar effects were seen in primary neurons at a much lower concentration of metformin (10 μM) (Fig. 1*B*). To ascertain that the intracellular Aβ measured from cell lysates did not include the secreted Aβ that is often associated with cell membranes, we briefly treated cells with trypsin and then with trypsin inhibitors before lysis and found no significant difference in the intracellular Aβ levels with or without trypsin cleavage (Fig. S1*A*).

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The authors declare no conflict of interest.

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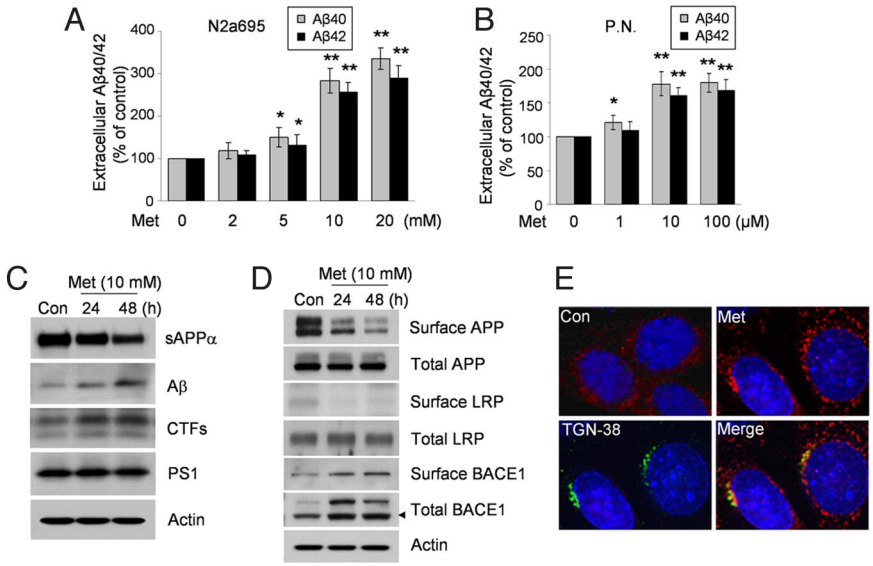

Fig. 1. Effects of metformin on APP/Aβ metabolism. (*A*) Dose-dependent effects on extracellular Aβ40/42 in N2a695 cells as measured by ELISAs. Data represented as means from 6 independent experiments. (*B*) Effects on extracellular Aβ40/42 in primary neurons. Data collected from 3 independent experiments. (*C*) Western analysis of APP metabolites, including intracellular Aβ, sAPPα, and CTFs upon metformin treatment. (*D*) Alterations of surface and total protein levels of APP, LRP and BACE1 upon metformin treatment. (*E*) Immunocytochemistry showing increased Aβ40 species in the *trans*-Golgi network colocalized with the TGN marker (TGN-38) in metformin-treated cells compared with the untreated control. Fluorescent images (100× magnification): green, TGN-38; red, anti-Aβ40.

We then analyzed the levels of various APP metabolites including the cleavage products of α- and β-secretases (Fig. 1*C*). Metformin reduced α-cleavage and promoted β-cleavage, as evidenced by decreased sAPPα and increased APP C-terminal fragment, CTF-β (the upper CTF band that resulted from cleavage by BACE1). No change in the levels of full-length PS1 (presenilin 1, the core component of γ-secretase) or its N-terminal fragment was detected from total cell lysates.

We also examined the potential effect of metformin on protein trafficking. The surface levels of both APP and LRP1 (low-density lipoprotein receptor-related protein 1), which are known to comigrate during trafficking, were dramatically reduced after metformin treatment as detected by biotinylation assays, whereas their total protein levels remained unchanged (Fig. 1*D*). However, the surface and the total BACE1 were markedly increased by metformin. Through further subfractionation, using sucrose gradients, we showed that metformin treatment caused changes in the compartmentalization of APP, as evidenced by increased distribution in *trans*-Golgi network (TGN) vesicles (fraction 2), including those trafficks en route to early endosomes and TGN (fraction 3), and decreased distribution in membranes (fraction 5) (Fig. S1 *B* and *C*). BACE1 protein levels were found to be elevated in all 3 fractions: 2, 3, and 5, with an ≈2-fold increase of the total protein. The increased distribution of both APP and BACE1 in fractions 2 and 3 are expected to favor Aβ generation within TGN and/or endocytic compartments, the two compartments with mild acidic pH optimal for BACE1 activity (33, 34). Indeed, we detected increased immunofluorescent Aβ40 signals after metformin treatment in TGN (Fig. 1*E*).

We next studied the potential effect of metformin on the levels of 2 enzymes known to degrade Aβ, neprilysin and insulin-degrading enzyme (IDE). Metformin had no effect on both enzymes, including protein levels and their activities (Fig. S2 *A* and *B*). Moreover, metformin had no effect on Aβ degradation as measured by pulse–chase assay (Fig. S2*C*). We also found that the increased Aβ production caused by metformin was not due to increased APP expression, because the total APP level was unaltered (Fig. 1*D*).

Metformin Up-Regulates BACE1 Promoter Activity. As correlated with its increased protein level (Fig. 1*D*), metformin increased the total BACE1 enzymatic activity by 2-fold (Fig. 2*A*). BACE1 mRNA was also increased by metformin in a time-dependent manner in both N2a695 and primary cortical neurons (Fig. 2*B*),

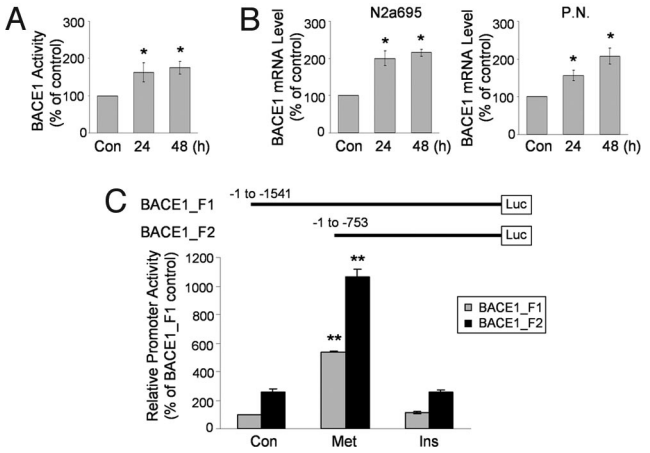

Fig. 2. Effects of metformin on BACE 1 expression and activity. Data presented as mean values from at least 3 independent experiments. (*A*) Metformin's effect on BACE 1 enzymatic activity as measured by Sigma's activity assay kit. Data therefore represent the relative total BACE1 activity per cell. (*B*) Effect of metformin on BACE1 mRNA level. The BACE1 transcript levels were determined by semiquantitative RT-PCR in both N2a695 and primary neurons. (*C*) Metformin up-regulates BACE1 promoter activity. Data represents the luciferase activity of a 1.5-kb BACE1 promoter-luciferase construct after transient transfection into N2a695 cells with or without treatment with metformin (10 mM) or insulin (1 μM) for 24 h. The BACE1 promoter activity is presented with respect to the activity of a control plasmid. The gray bars represent the relative luciferase activity with full-length BACE1 promoter (BACE_F1), whereas the black bars represent the activity of the truncated promoter (BACE1_F2) lacking the first 3 PPAR/RXR binding elements as illustrated in the scheme above the bar graph. In both settings, metformin up-regulates the promoter activity of BACE1_F1 and BACE1_F2 to a similar degree, suggesting a PPARγ-independent mechanism. $n = 5$.

Table 1. Predicted RXR/PPAR binding elements in BACE1 promoter region

Family	Matrix	Position	Strand	Sequence
V$RXRF	V$PXR_RXR.01	−1357/−1333	+	caggtgGGTCatgaggattcatatc
V$PERO	V$PPAR_RXR.01	−1239/−1217	+	tccctgttggatacTGGTgagtg
V$RXRF	V$CAR_RXR.01	−1070/−1046	−	gttcaaGGACagccaagactacata
V$RXRF	V$CAR_RXR.01	−398/−374	−	tgctcaGGCCaccataatccagctc

The putative PPAR/RXR heterodimer binding sites in the rat *BACE1* promoter predicted by the online program MatInspector (www.genomatix.de). V$ represents the vertebrate family. The capital letters in the sequence represent core sequence, and the underlined regions represent ci-value > 60, according to matrix family assignment with the RXR consensus sequences. The adenine+1 represents the translational start site.

as measured by semiquantitative RT-PCR. A luciferase reporter assay on a 1.5-kb *BACE1* promoter (35) showed that metformin increased promoter activity by >5-fold whereas insulin had no effect (Fig. 2*C*).

Recently, *BACE1* promoter activity was reported to be modulated by PPARγ-dependent transactivation. In addition to the PPARγ-responsive element (PPRE) identified (36), 3 additional binding sites for RXR heterodimers were predicted within the 1.5-kb *BACE1* promoter based on their consensus motifs (Table 1). We therefore examined whether metformin up-regulates *BACE1* transcription through a PPARγ-RXR-mediated pathway using a luciferase reporter construct containing a 5′ truncated fragment of the rat *BACE*1 promoter (−1, −753). This truncated promoter lacked the first 3 PPRE/RXR elements but still exhibited 5-fold-increased activity upon metformin treatment (Fig. 2*C*), suggesting an up-regulation of *BACE1* transcription independently of PPARγ.

Metformin's Effect Is Independent of Glucose Metabolism and Insulin Signaling. To investigate whether the Aβ-increasing effect of metformin depends on insulin levels and glucose metabolism, metformin-treated N2a695 cells were cultured in low-glucose media or in serum-free conditions. Under low glucose conditions (5 mM glucose for 24 h) Aβ production was slightly reduced (Fig. 3*A*). However, metformin still increased Aβ production to a similar degree as normal conditions (25 mM glucose). In contrast, cells cultured under serum-free conditions for 24 h, where the basal intracellular Aβ level was reduced by 30%, became completely resistant to metformin treatment. This result suggests that the effect of metformin depends on the presence of growth factors in the serum, possibly including insulin and insulin-like growth factors (IGFs).

To determine whether insulin signaling is involved in mediating metformin's action, we tested several inhibitors to tyrosine receptor protein kinase, including 2 potent pan-tyrphostins (A25, AG126) and the insulin/IGF-1 pathway-selective AG538. Interestingly, all 3 tyrphostins did not show any significant effect at the concentrations tested (1–25 μM; Fig. 3*B*). Taken together, these results indicate that metformin likely augments Aβ production through mechanisms independent of insulin signaling and glucose metabolism.

Metformin's Effect Is Mediated by Activation of the AMP Kinase (AMPK) in Vitro and in Vivo. We examined whether metformin's Aβ-increasing effect depended on activation of AMPK, a known molecular target of metformin. Phosphorylation of AMPK at Thr-172 and its substrate, acetyl CoA carboxylase (ACC), were found to be both induced by metformin in a dose-dependent manner (Fig. 4 *A* and *B*). We also observed a significant inhibition of metformin-stimulated Aβ production by compound C, a specific AMPK inhibitor, in a dose-dependent manner. Compound C inhibited metformin's effect by 50% when used at a concentration (20 μM) that is known to guarantee its specificity for AMPK (32) (Fig. 4*C*). These results indicate an AMPK-dependent mechanism for metformin's effect on Aβ. Significantly, the antagonizing effect of compound C was largely

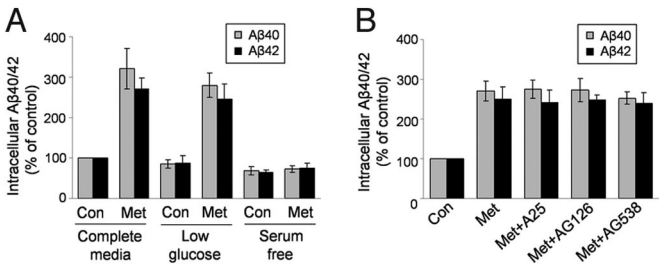

Fig. 3. Metformin's effect is independent of glucose levels and insulin signaling. (*A*) For low glucose conditions, cells were cultured in low-glucose DMEM (5 mM) overnight and then metformin was added for an additional 24 h in this condition compared with normal glucose (25 mM). For serum-free conditions, cells were cultured in DMEM/Opti-MEM for 24 h. Intracellular Aβ production was measured by ELISA of lysates (diluted 50-fold) collected from cells after the last 4 h incubation in serum-free media. (*B*) Effects of various tyrphostins on metformin's modulation of Aβ levels. Cells were pretreated with metformin for 24 h and inhibitors were added at 10 μM concentrations the second day after switching to serum-free media. Aβ ELISAs were performed using cell lysates collected 4 h after incubation with various inhibitors. $n = 4$.

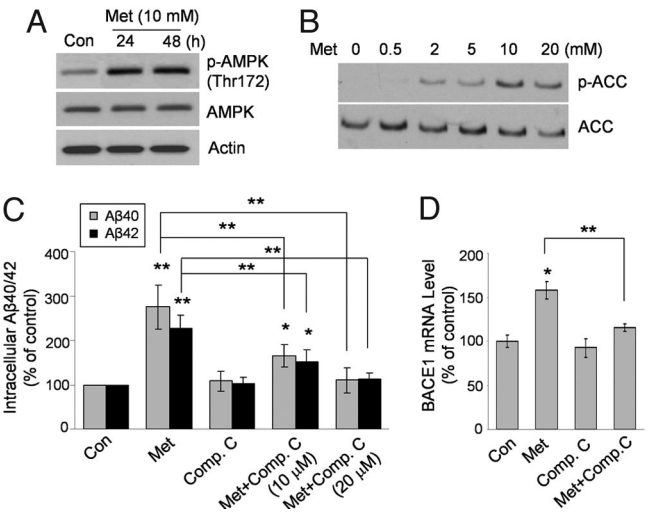

Fig. 4. Metformin's effect depends on AMPK activation. (*A*) Metformin activates AMPK in N2a695 cells. Western blot analysis shows a marked elevation of the Thr-172 phosphorylated AMPK. (*B*) Dose-dependent effect of metformin on activating ACC, the AMPK downstream substrate, as measured by Western blot analysis of the phosphorylated ACC (Ser-79). (*C*) Effect of the AMPK inhibitor Compound C (Comp. C) on abolishing metformin's effect on Aβ levels. Comp. C completely abolishes the effect of metformin on intracellular Aβ production as measured by ELISA of cell lysates (diluted 50-fold). (*D*) Combinatory effects of metformin and Comp. C on *BACE1* transcription as determined by semiquantitative RT-PCR.

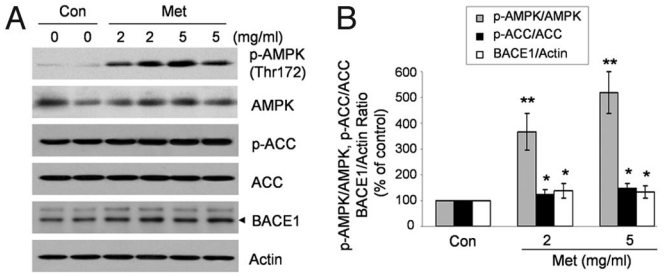

Fig. 5. Metformin activates AMPK/BACE1 in WT C57B6 mice. (*A*) Western analysis of phosphorylated AMPK, phosphorylated ACC and BACE1 protein levels in mouse brain lysates (frontal region) after receiving metformin in drinking water for 6 days. $n = 4$ animals in each groups. (*B*) The bar graph shows quantitative data of *A*.

attributed to suppression of *BACE1* transcription because the mRNA was greatly reduced after treatment with the two drugs (Fig. 4*D*).

To validate our observations obtained from neuronal culture systems, we treated C57B6 mice with metformin (2–5 mg/mL in drinking water) for 6 days and found that its activating effects on AMPK/ACC and on BACE1 were valid in mouse brains (frontal region) (Fig. 5), indicating that the drug exerts a similar effect in vitro and in vivo. Pharmacological analysis, using LC-MS, indicated that metformin treatment at 2 mg/mL for 6 days in mice resulted in accumulation of the drug to ≈1 μM concentration in the brain (Table S1), suggesting that metformin likely exerts a direct effect on APP processing in neurons via activating the AMPK pathway, as was suggested by neuronal models. Indeed, we also observed a similar augmenting effect of metformin on Aβ levels in a transgenic mouse line after drinking 2 mg/mL drug for 3 months (Fig. S3).

Antagonizing Effect on Intracellular Aβ Generation. Because metformin is known to sensitize insulin's effects, one would expect that the two drugs may exert similar or even synergistic effects on APP/Aβ metabolism. Nevertheless, we reported that 1 μM insulin reduces intraneuronal Aβ by accelerating APP trafficking and inhibiting Aβ degradation (29). Consistent with our previous finding, we observed a significant reduction of intracellular Aβ in the presence of 1 μM, but not 0.25 μM, insulin. Interestingly, metformin not only failed to increase intracellular Aβ, but also potentiated insulin's Aβ-lowering effect, with a significant inhibition of intracellular Aβ at 0.25 μM insulin, when both insulin and metformin (10 mM) were present (Fig. 6).

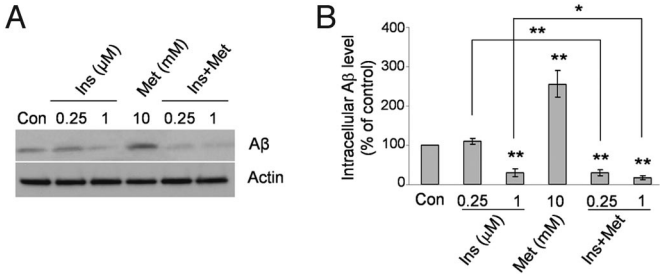

Fig. 6. Combination of insulin and metformin reduces Aβ generation. (*A*) N2a695 cells were pretreated with 10 mM metformin for 24 h, and exogenous insulin was added at the 2 concentrations (0.25 μM or 1 μM) in serum-free media for an additional 4 h in combination with metformin. Intracellular Aβ levels were measured by IP-Western analysis. To measure insulin's effect, it was added directly to the cultures in serum-free media for 4 h before the Aβ assays. (*B*) The bar graph shows quantitative data of the representative IP-Western blot of *A*. $n = 3$.

Discussion

BACE1 is the predominant neuronal β-secretase, catalyzing β-cleavage of APP. Both its protein level and enzymatic activity are elevated in AD brains, suggesting that abnormal BACE1 regulation may contribute significantly to AD pathogenesis. Several transcriptional factors have been identified that modulate *BACE1* transcription and some are involved in the inflammatory and chronic stress responses in the brain that are compromised during aging. For instance, *BACE1* is up-regulated in neurons by (*i*) oxidative stress, (*ii*) chronic models of gliosis, (*ii*) experimental traumatic brain injury, and (*iv*) hypoxia conditions as recently demonstrated by our group and others (see ref. 2 for review).

BACE1 transcription has recently been reported to be regulated by the PPARγ pathway (36). We now demonstrate that the diabetes drug metformin can also modulate BACE1 transcription, likely independently of the PPARγ pathway despite the presence of several PPAR/RXR binding sites in the promoter (31, 32). Metformin-mediated transcriptional activation of *BACE1* appears to depended on a pathway involving AMPK. Recently, the AMP-activated protein kinase (AMPK) has been identified as one of the molecular targets of metformin, accounting for the majority of its pleiotropic effects. AMPK, which acts as a fuel-sensing enzyme in glucose and fatty acid metabolism, is ubiquitously expressed and highly conserved in the eukaryotic kingdom (37, 38). Because AMPK is activated by metformin in parallel with the up-regulation of Aβ generation in our experimental systems, and is antagonized by the AMPK inhibitor compound C (Fig. 5), it is likely that metformin modulates *BACE1* transcription through activating a signaling pathway, at least in part, involving AMPK.

Our finding that metformin increases Aβ generation and secretion raises the concern of potential side-effects, of accelerating AD clinical manifestation in patients with type 2 diabetes, especially in the aged population. This concern needs to be addressed by direct testing of the drug in animal models, in conjunction with learning, memory and behavioral tests. Although the precise mechanism of action of metformin in the CNS remains largely unknown, recent studies (39, 40), together with ours, suggest that metformin crosses the blood brain barrier and exerts specific pharmacological effects in rodent brains upon chronic administration. Indeed, our study using a comparable dose (2 mg/mL is equivalent to a clinical dosage of 300 mg/kg/day) indicates that systemically administrated metformin for 6 days increases brain AMPK activation and BACE1 protein levels (Fig. 5). A direct measurement of the metformin concentration in these mice showed that it reaches 2 μM and 1 μM in the plasma and forebrain region, respectively (Table S1); Considering that the steady-state plasma level of metformin in patients is reported to be from 10 μM to as high as 40 μM (32) and we achieved the maximum effect of metformin on BACE1 levels and AMPK activation in primary cultured neurons at 1–10 μM, the potential side-effects of metformin on accelerating AD pathogenesis must be taken seriously.

Although the study was performed in neuronal models, metformin appears to be able to sensitize/enhance insulin's anti-Aβ effect as it can lower the effective concentration of insulin (Fig. 6), which agrees with its insulin-sensitizing effect in other settings (37, 38). It remains as an interesting question how metformin specifically sensitizes insulin's effect on reducing Aβ generation while diminishing its own stimulatory effect, when used together. We believe that the combinatory effect of metformin and insulin, as shown in Fig. 6, involves the interplay of their antagonizing effects on *BACE1* transcription and on APP processing/trafficking. Additional mechanisms may also be involved because insulin signaling has multiple complex effects in the CNS. The levels of insulin, insulin receptors and IDE are

reduced in AD brains (41, 42). Furthermore, a specific inhibitory effect of soluble Aβ on insulin signaling has also been reported (43, 44). Because the neurotoxicity of intraneuronal soluble forms of oligomeric Aβ species to synaptic functions has been increasingly reported, it is important to determine the specific Aβ species that are regulated by metformin/insulin. We have conducted preliminary studies on the brains taken from triple transgenic mice (45) after a 3-month metformin treatment (4–7 months) and found a significant increase in BACE1 levels and soluble Aβ (Fig. S3).

Our data suggest that the potentially deleterious effects of metformin to AD patients may be avoided by using it in combination with insulin; the combination may result in a beneficial effect in treating both type 2 DM and in mitigating AD progression. Despite the strong link between DM and AD, the association between DM and the neuropathology of AD is less clear, based on a few conflicting reports on limited patient cohorts/populations (46–49). Studying the interaction between medications for DM and AD neuropathology may clarify the relationships between diabetes, diabetes medications, and AD. In particular, comprehensive studies that include a large patient cohort that take various diabetes medications (monotherapy with insulin or oral drug versus combination therapy) are needed. Indeed, two recent reports found significantly fewer neuritic plaques (NPs) in the brains of diabetics taking a combination of insulin and oral drugs, compared with those taking a single drug (insulin or oral medicine) (50, 51), which supports our observations (Fig. 6), although those patients taking metformin alone should be further evaluated for AD neuropathology and Aβ content in CSF.

Likewise, it is probably beneficial to combine metformin with certain TZDs based on the known suppressive role of certain PPARγ agonists in BACE1 transcription (36). The distinct but complementary mechanisms of action of these two drug types in insulin-sensitization, the reduction of inflammation and in lowering the risk of cardiovascular diseases, support the rationale for TZD/metformin combination therapy in patients with type 2 diabetes. In fact, the use of a single pill containing metformin and rosiglitazone (Avandamet) was approved by the FDA in October 2002 for the treatment of diabetes (52). Given the recent reported beneficial effects of certain TZD in attenuating learning and memory deficits in AD mouse models (53, 54), it remains to be determined whether a complementary effect for metformin and TZD can be achieved to benefit the patients with DM and AD.

Materials and Methods

Chemicals and Antibodies. Metformin was obtained from 2 sources (Sigma–Aldrich and Calbiochem). Insulin and tyrphostins AG538 and AG126 were obtained from Sigma–Aldrich. The monoclonal antibody 6E10 and the ELISA kits for Aβ 40 and Aβ 42 were obtained from Signet Laboratories. The polyclonal antibodies against the C-terminal portion of APP (369) and CT-BACE1 were developed in our laboratory. Anti-AMPK, ACC and their phospho-antibodies were obtained from Cell Signaling. Anti-IDE 28H1 clone was from Santa Cruz Biotechnology; anti-neprilysin and 22C11 monoclonal antibody were from Millipore. Compound C, anti-β-amyloid 40 (FCA3340) and anti-β-amyloid 42 (FCA3542) were obtained from Calbiochem.

Cell Lines and Culture Treatment. N2a695 cells were maintained in DMEM. Mouse P0 primary cortical cultures were prepared as described in ref. 29. For drug treatments, cells were pretreated with metformin for 24 h and then switched to serum-free media for an additional 4 h before the cultured supernatants were collected for Aβ ELISAs. For the inhibitors, compound C or tyrphostins were added alone in serum-free conditions for 4 h or after pretreatment of cells with metformin for 24 h.

IP-Western Analysis and Immunocytochemistry. The IP-Western was performed as described to detect intracellular Aβ (29). Double fluorescent staining was performed as described in ref. 33, using specific rabbit anti-Aβ 40 (FCA3340) and mouse anti-TGN38 (BD Transduction Laboratories), both at 1:500 dilution.

Cell Surface Biotinylation. Biotinylation was performed using sulfo-NHS-LC-biotin (Pierce), which was added to cultures at 0.5 mg/mL for 1 h at 4 °C. After washes, cells were lysed with Nonidet P-40 lysis buffer. Biotinylated cell surface proteins were immunoprecipitated by anti-streptavidine-beads and the surface APP (or BACE1) was detected with specific antibodies (22C11 or CT-BACE1) by Western blot analysis.

RT-PCR on BACE1 Messages. Total RNA was extracted using TRIzol reagent (Invitrogen). SuperScript First-Strand kit (Invitrogen) was used to synthesize the first strand cDNA from samples with an equal amount of RNA, according to the manufacturer's instructions. Synthesized cDNAs were amplified using IQ SYBR green supermix (Biopioneer) and ICycler from Bio-Rad; data were analyzed using Bio-Rad MyIQ 2.0. Three pairs of primers used for *BACE1* amplification are listed below. The pair of mouse specific primers were: forward, 5′-GATGGTGGACAACCTGAG-3′, and reverse, 5′-CTGGTAGTAGCGATGCAG-3′. The rattus primers used were: forward, 5′ TTGCCCAAGAAAGTATTTGAAG 3′, and reverse, 5′ CGGAAGGACTGATTGGTG 3′. Primers used for GAPDH amplification were: GAPDH-5, 5′-CGTGGAGTCTACTGGTGTC-3′ and GAPDH-3, 5′-ATCATACTTGGCAGGTTTCTC-3′. BACE1 mRNA levels were normalized to levels of GAPDH.

Promoter Activity by Luciferase Assays. We cloned the 1.5-kb segment of the rat *BACE1* promoter into pGL3-Basic vector containing the firefly luciferase gene (Promega) via PCR, using genomic DNA from PC12 cells as a template and the primers as described in ref. 31. The sequence-confirmed constructs were transfected into N2a695 cells, using Lipofectamine 2000 (Invitrogen) and pRL-CMV containing the Renilla luciferase gene (Promega) was cotransfected as an internal control. The transfected cells were treated with or without 10 mM metformin or 1 μM insulin for 24 h. The luciferase reporter assay was performed according to the manufacturer's instructions (Promega).

Statistical Evaluation. All statistical analysis was performed by ANOVA, followed by Dunnett or Tukey–Kramer post hoc tests. Data are means ± SD. The asterisks indicate significant difference versus control as follows: *, $P < 0.05$ and **, $P < 0.01$.

For more information, please see *SI Methods*.

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