Increased Aβ production prompts the onset of glucose intolerance and insulin resistance

Margarita Jiménez-Palomares,1 Juan José Ramos-Rodríguez,2 José Francisco López-Acosta,1 Mar Pacheco-Herrero,2 Alfonso M. Lechuga-Sancho,1,3 Germán Perdomo,1 Mónica García-Alloza,2 and Irene Cózar-Castellano3

1Unidad de Investigación, Hospital Universitario Puerta del Mar, Cadiz, Spain; 2Área de Fisiología, Universidad de Cádiz, Cádiz, Spain; and 3Área de Pediatría, Departamento Materno-Infantil y Radiología, Universidad de Cádiz, Cádiz, Spain

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ALZHEIMER’S DISEASE (AD) and type 2 diabetes (T2D) are two prevalent diseases in developed countries with comparable pathological features and genetic predisposition (3, 4, 7). Several clinical and epidemiological studies have shown a relationship between AD and T2D. Patients with T2D exhibit an increased risk for developing dementia and AD (1, 11, 12, 14, 15, 19). On the other hand, patients suffering AD are more vulnerable to develop T2D (10). Patients with AD show a remarkable deposition of β-amyloid peptide (Aβ) in brain, whereas patients with T2D present islet amyloid polypeptide deposition in pancreatic β-cells (6). On the other hand, patients with T2D are glucose intolerant and insulin resistant, revealing decreased insulin signaling in peripheral tissues, whereas patients with AD show signs of impaired insulin signaling in the brain (16). Taken together, these observations have spurred the hypothesis that these pathological alterations may underlie the mechanistic link between T2D and AD.

To elucidate the underlying molecular mechanism linking T2D and AD, Takeda et al. (18) developed two mouse models of AD and diabetes. In the first model, they crossed an animal model for AD (APP23-transgenic mice) that expresses human type amyloid precursor protein (APP), with leptin deficient db/db mice as a model for T2D and obesity. In the second model, they crossed APP23 mice with Nagoya-Shibata-Yasuda (NSY) mice, a model of T2D with mild obesity. They demonstrated that diabetic predisposition exacerbated cognitive dysfunction, which was associated with cerebrovascular inflammation and cerebral amyloid angiopathy in both mouse models. In addition, brain insulin signaling was impaired, which was associated with neuropathological changes (18). However, this study does not demonstrate completely whether AD could accelerate the development of the diabetic phenotype.

To further clarify the relationship between AD and T2D, we investigated the underlying mechanisms linking AD and T2D with an alternative approach. We hypothesized that genetic predisposition for developing AD would prompt the onset of T2D. To test this hypothesis, we crossed APPswe/PS1dE9 (APP/PS1) transgenic mice, a well established animal model of AD, with heterozygous leptin receptor-deficient mice (db/+), a mouse model for genetic predisposition of T2D without diabetic metabolic traits. We showed that APPswe/PS1dE9 mutations prompted the onset of glucose intolerance and insulin resistance in db/+ mice. Furthermore, these metabolic alterations of glucose homeostasis were paralleled with augmented pancreatic β-cell mass and hyperinsulinemia. Thus, our results provide new insights into the mechanisms underlying the pathophysiology of AD and T2D.

MATERIALS AND METHODS

Ethical approval. Experimental procedures were approved by the Animal Care and Use Committee of the University of Cadiz in accordance with the Guidelines for Care and Use of Mammals in Research (European Commission Directive 86/609/CEE and Spanish Royal Decree 1201/2005).

Experimental mice. The mouse model of obesity and type 2 diabetes used in this study is the db/db mouse. C57BL/KsJ heterozygous db/+ mice were purchased from Harlan Laboratories (Boxmeer, The Netherlands). We used the APPswe/PS1dE9-line 85 as a mouse model of AD. These mice coexpress a chimeric mouse/human APP695 harboring the Swedish K670M/N671L mutations (M/
HuAPPswe) and human PS1 with the exon-9 deletion mutation (PS1dE9) (9). The two transgenes co-integrate and segregate as a single locus. APPswe/PS1dE9 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were fed standard rodent chow diet and water ad libitum in ventilation-controlled cages under a 12:12-light-dark cycle.

The experimental cohorts used in this study were males and females of the F2 generation. The F1 generation was composed of heterozygous db/+;APPswe/PS1dE9 from crosses of db/+ and APPswe/PS1dE9 in the parental generation. Twelve independent cohorts were used for the metabolic studies and collection of tissues.

Mice were bred at the animal facility of the University of Cadiz, Cadiz, Spain.

**Mouse genotyping.** Wild-type (WT), db/db and db/+ mice were generated from crosses between heterozygous db/+ mice. The introduction of an Rsal site by the Lepr^ab mutation in the leptin receptor gene was detected by PCR. Briefly, genomic DNA from mouse tail was isolated using GenElute Mammalian Genomic DNA Miniprep Kit (Sigma, St. Louis, MO) according to the manufacturer’s instructions, followed by a PCR reaction to amplify a 135-bp fragment spanning the Lepr^ab mutation (primers: Lepr forward, 5'-AGAACGGAGCAGCTTGGAGTGGAACA-3'; Lepr reverse, 5'-CATTCAACACATAGTGTAGGTTGTTTGCT-3'). Afterward, a total of 20 µL of PCR product was digested by Rsal (Invitrogen Europe) and resolved in 10% polyacrylamide gels. Mice carrying the homozygous Lepr^ab mutation produced fragments of 108 and 27 bp, while heterozygous mice produced fragments of 135, 108, and 27 bp, and WT mice produced a fragment of 135 bp.

APP/PS1 mice were generated from crosses between WT and hemizygote transgenic mice. Mouse genotyping were performed as described previously (8) with some modifications. Briefly, genomic DNA from mouse tail was isolated as described above, followed by a PCR reaction to amplify a 760-bp fragment in WT mice, whereas transgenic mice produced fragments of 760 and 240 bp (primers: 1,501, 5'-CTGACCACTCGACCAGGTTCTGGGT-3'; 1,502, 5'-GTGGAATAACCCTCCTCCAGGCCTAGACCA-3'; and 1,503, 5'-CTGACACACTCGCAAGGGTTCGTTGCT-3').

**Determination of Aβ levels in brain and plasma.** Immunohistochemistry for Aβ was performed on 30 µm of paraformaldehydefixed brain sections. Sections were washed in TBS and pre-treated with formic acid 70% for 30 min at room temperature. Then they were blocked in 5% normal goat serum with 0.5% Triton-X for 1 h. Fixed tissues were washed extensively in water and immersed in Bouin’s solution (Sigma-Aldrich) for 4 h. Tissues were removed and fixed in Bouin’s solution (Sigma-Aldrich) for 4 h. Tissues were dehydrated and paraffin embedded. Two standard paraffin sections of 5 µm of pancreas were labeled with guinea pig anti-insulin antibody (1:200 dilution overnight at 4°C; Cell Signaling Technology) and antibodies against p-Akt (Ser473) and Akt antibodies (1:1,000 dilution overnight at 4°C; Cell Signaling Technology) and secondary antibody anti-rabbit (1:5,000 dilution, 30 min; Jackson ImmunoResearch) were used.

**Quantitative real-time PCR.** Total RNA was isolated from liver or skeletal muscle tissues using the RNeasy Plus Micro Kit (Qiagen) followed by DNase treatment. cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis kit (Roche). The real-time quantitative PCR reaction was performed from cDNA using SYBR Premix Ex Taq (Perfect Real Time) according to the manufacturer’s protocol (Takara Bio). The primer sets used were as follows: phosphoenolpyruvate carboxykinase (PEPCK) forward (5'-TCA-GCTGCATAACAGGCTTGCTTGCAAC-3'), PEPCK reverse (5'-GCTTTGTTCTCAGAATCGTCTGCTT-3'); glucose-6-phosphatase (G-6-Pase) forward (5'-GGAGAAAATGACACATTCT-3'), G-6-Pase reverse (5'-GGTCCTGCTCACCAGAGTAC-3'); 18s ribosomal RNA (18S) forward (5'-GAACGGCTACCAATTCGAG-3'), 18s reverse (5'-GCCCTCAATGGATCTCCCTCTGTT-3'). Data are presented as the fold change in PEPCK, and G-6-Pase gene expression was normalized to the levels of the housekeeping gene 18S.

**Plasma biochemistry.** Blood glucose was obtained from mice under fasting (16 h) or nonfasting conditions (free access to food pellets). Blood glucose levels were measured from nicked tails using the glucometer Optium Xceed (Abbott). Blood for plasma insulin and cholesterol determination was collected from the tail vein into capillary tubes precoated with potassium-EDTA (Sarstedt, Nümbrecht, Germany). Plasma insulin levels were measured using ultrasensitive mouse enzyme-linked immunosorbent assay (ALPCO Diagnostics, Salem, NH). Plasma cholesterol levels were determined using the Wako cholesterol reagent (Wako Chemicals, Neuss, Germany).

**Intraperitoneal glucose tolerance test.** To evaluate alterations in glucose homeostasis in vivo, we performed a glucose tolerance test (GTT). Mice were fasted for 16 h and injected intraperitoneally with glucose at 2 g/kg body wt. Blood glucose levels were determined and plotted as a function of time. The area under the curve was calculated using an equation that quantifies the incremental area above the baseline, where only the area above the fasting level for each mouse is taken into account.

**Intraperitoneal insulin tolerance test.** To evaluate the insulin action in vivo, we performed an insulin tolerance test (ITT). Mice were injected intraperitoneally with human insulin (Humulin; Eli Lilly) at a dose of 0.75 U/kg body wt. The rate of fall of blood glucose levels was monitored before and at different times after insulin injection. For each individual mouse, blood glucose levels were presented as the percentage of basal glucose concentration and plotted as a function of time. The area under the curve was calculated as described above.

**Western blot.** Mice were starved overnight, with insulin (Humulin) injected intraperitoneally at a dose of 0.75 U/kg body wt, and euthanized 15 min after insulin injection. Liver, soleus, and gastrocnemius muscles were removed and dipped in liquid nitrogen.

Tissues were homogenized in protein extraction buffer (Cell Signaling Technology) and proteins quantified by the Micro BCA kit (Thermo Scientific). Protein extracts were run in 10% polyacrylamide gels and transferred to polyvinylidene difluoride, p-Akt (Ser473) and Akt antibodies (1:1,000 dilution overnight at 4°C; Cell Signaling Technology) and secondary antibody anti-rabbit (1:5,000 dilution, 30 min; Jackson ImmunoResearch) were used.

**Effect of APP/PS1 transgenic expression on glucose homeostasis.** A neuropathological feature of AD is increased Aβ production and secretion in neurons. Transgenic APP/PS1 mice overexpress Aβ, which is detected in circulation (~300 pg/mL). To evaluate the impact of increased Aβ production on glucose
metabolism, we characterized transgenic APP/PS1 mice at 5–7 wk of age.

Transgenic APP/PS1 mice displayed normal body weight (Fig. 1A), blood glucose (Fig. 1B), and plasma insulin levels (Fig. 1C) compared with control littermates. Likewise, body weight, nonfasting blood glucose, and nonfasting plasma insulin at 10 and 32 wk of age were not different between APP/PS1 mice and their controls (Fig. 1, D–F). Finally, transgenic APP/PS1 mice exhibited similar glucose (Fig. 2, A and B) and insulin tolerance (Fig. 2, C and D) than control littermates at 7 wk of age.

Taken together, these results suggest that increased Aβ production per se does not alter glucose homeostasis or insulin sensitivity.

Effect of APP/PS1 transgenic expression on glucose homeostasis in db/db mice. Because we did not detect any metabolic phenotype on glucose homeostasis or insulin sensitivity in APP/PS1 mice, we investigated whether coexpressing APP/PS1 in a genetic background prone to develop obesity and T2D would alter glucose homeostasis. To this end, we used the db/db mouse model of obesity and T2D. The db/db mouse develops obesity, glucose intolerance, insulin resistance, and diabetes at 6–7 wk of age. However, the heterozygous db/+ mouse does not develop any metabolic phenotype. Thus, we characterized the glucose homeostasis in mice coexpressing APP/PS1 in a genetic background prone to developing T2D and obesity (db/+; APPswe/PS1dE9) compared with control mice (db/+; WT). Interestingly, although db/+; APP/PS1 mice did not show brain Aβ plaques at 5–7 wk of age (Fig. 3A), they did show increased expression of Aβ in plasma compared with db/+; WT (Fig. 3B).

As shown in Fig. 4A, coexpression of APP/PS1 genes in db/+ male and female mice at 5–7 wk of age did not alter body weight (Fig. 4A). Likewise, fasting blood glucose levels remained unchanged between control and db/+; APP/PS1 mice (Fig. 4B). In contrast, nonfasting blood glucose levels were elevated significantly in db/+; APP/PS1 compared with control mice (Fig. 4B). Interestingly, nonfasting hyperglycemia paralleled with nonfasting and fasting hyperinsulinemia in db/+; APP/PS1 mice (Fig. 4C). Finally, db/+; APP/PS1 mice showed elevated nonfasting plasma cholesterol levels compared with control mice, whereas fasting cholesterol levels remained unchanged (Fig. 4D). Taken together, these results indicate that Aβ overproduction alters glucose homeostasis in mice with a genetic predisposition to develop T2D.

Fig. 1. Increased β-amyloid peptide (Aβ) production in APPswe/PS1dE9 mice does not alter body weight or glucose metabolism. The effect of APPswe/PS1dE9 transgenic expression on body weight and glucose metabolism was investigated in normal mice at 5–7 wk of age in nonfasting or fasting state. A: body weight in females and males. B: blood glucose levels in nonfasting and fasting conditions. C: plasma insulin levels in nonfasting and fasting conditions (n = 15–16/genotype). D: body weight at 10 and 32 wk of age. E: blood glucose levels in nonfasting conditions at 10 and 32 wk of age (n = 7–9/genotype). F: plasma insulin levels in nonfasting conditions at 10 and 32 wk of age (n = 7–9/genotype). WT, wild type.
end, we performed intraperitoneal (IP) GTT and IPITT in db/H11001;APPswe/PS1dE9 and control mice (db/H11001;WT). As shown in Fig. 5, A and B, db/H11001;APP/PS1 mice exhibited impaired glucose tolerance compared with control mice. In addition, we measured glucose-stimulated insulin release during the glucose tolerance test. As expected, db/H11001;APP/PS1 mice exhibited higher plasma insulin levels than control mice immediately before (time 0) glucose injection (Fig. 5, C). However, after 15 min (time 15) of glucose injection, plasma insulin levels were similar between groups (Fig. 5, C). Interestingly, db/H11001;APP/PS1 mice showed decreased insulin sensitivity compared with control mice (Fig. 5, D and E). Thus, our results indicate that Aβ overproduction is associated with glucose intolerance and insulin resistance in db/H11001 mice.

In physiological conditions during the postprandial state, insulin inhibits hepatic gluconeogenesis, and consequently hepatic glucose output is abolished. In pathophysiological conditions of hepatic insulin resistance, insulin is unable to suppress hepatic gluconeogenesis; thus hepatic glucose output goes awry, contributing to elevate postprandial blood glucose levels. Hence, hepatic insulin resistance is a major contributor to hyperglycemia and T2D. To further investigate the insulin resistance observed in db/H11001;APP/PS1 mice, we tested peripheral insulin signaling (Akt phosphorylation) in skeletal muscle, gastrocnemius (Fig. 6, A and B) and soleus (Fig. 6, C and D), and liver (Fig. 6, E and F). Interestingly, whereas db/H11001;WT mice exhibited activation of the insulin-stimulated Akt pathway, db/H11001;APP/PS1 mice did not show activation of insulin signaling (Fig. 6). In the same sense, we also studied the mRNA levels of two enzymes responsible for hepatic gluconeogenesis (PEPCK and G-6-Pase; Fig. 6G). PEPCK and G-6-Pase mRNA levels were downregulated by insulin in the liver of db/H11001;WT mice, although it was statistically significant only for G-6-Pase. In contrast, insulin was unable to downregulate the expression levels of PEPCK and G-6-Pase in the liver of db/H11001;APP/PS1 mice. Taken together, these results support the notion that hepatic insulin resistance is a contributing factor to elevate postprandial blood glucose levels in db/H11001;APP/PS1 mice.
Transgenic APP/PS1 coexpression increases β-cell mass in \( db/\bar{+} \) mice. Under conditions of insulin resistance, the pancreatic β-cells compensate by increasing insulin secretion in an effort to sustain normoglycemia. Two different mechanisms are involved in β-cell compensation: enhancing cellular secretory capacity and/or increasing β-cell mass. As we showed above, glucose-stimulated insulin secretion was not altered in \( db/\bar{+} \); APP/PS1 mice (Fig. 5C); however, insulin secretion in basal conditions is increased in \( db/\bar{+} \); APP/PS1 mice with respect to their controls. To elucidate the cause of increased insulin secretion, we performed a histomorphometric analysis of β-cell mass in \( db/\bar{+} \); APP/PS1 and control mice (\( db/\bar{+} \); WT) to determine the impact of transgenic coexpression of APP/PS1 transgenes on β-cell mass. Pancreas weight in the two groups was not significantly different (data not shown). As shown in Fig. 7, β-cell mass and β-cell area were elevated significantly in \( db/\bar{+} \); APP/PS1 compared with \( db/\bar{+} \); WT mice (Fig. 7, A–C). In contrast, pancreatic islet number remained unchanged between \( db/\bar{+} \); APP/PS1 and \( db/\bar{+} \); WT mice (Fig. 7D). These results suggest that β-cell mass expansion is at least one mechanism by which \( db/\bar{+} \); APP/PS1 mice compensate for insulin resistance.

**DISCUSSION**

Epidemiological, clinical, and basic studies have shown a relationship between AD and T2D. Despite advances in our knowledge, definitive molecular mechanisms involved in the pathophysiology observed in both diseases remain unrevealed. In this study, we hypothesized that AD would prompt the onset of T2D. To test this hypothesis, we have developed a new mouse model crossing the APPswe/PS1dE9 mouse, a well-established AD mouse model, into the \( db/\bar{+} \) mouse back-
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Using this new model we were able to show that Aβ overproduction prompted the early onset of metabolic perturbation characteristics of T2D, such as glucose intolerance, insulin resistance, and pancreatic β-cell mass expansion. To our knowledge, this is the first study to show that AD accelerates the development of diabetic phenotype in mice.

This new model of AD and T2D is characterized by several features. First, we used a well-characterized AD mouse model.
coexpressing mouse/human amyloid precursor protein (APP695) with the Swedish K670M/N671L mutation and human presenilin 1 with the exon-9 deletion mutation (PS1dE9). Second, we used the heterozygous leptin receptor-deficient mouse (db/+). Interestingly, these mice are partially deficient in leptin signaling but do not display obesity, glucose intolerance, or insulin resistance compared with wild-type mice. Furthermore, insulin signaling remains intact in db/+ mice. Thus, we were able to investigate the effect of Aβ overexpression on glucose homeostasis and insulin sensitivity before the onset of obesity and T2D.

Other mouse models have been developed to investigate the pathophysiological interaction between T2D and AD. Takeda et al. (18) crossed the APP23 transgenic mice, a well-established AD mouse model, with leptin-deficient mice (ob/ob) or polygenic NSY mice as a model of diabetes mellitus. They demonstrated that the onset of diabetes exacerbated Alzheimer-like cognitive dysfunction in APP+/−ob/ob mice in addition to cerebrovascular inflammation and severe amyloid angiopathy. On the other hand, APP+/−ob/ob mice showed an accelerated diabetic phenotype compared with ob/ob mice. Likewise, NSY mice overexpressing APP23 exhibited severe glucose intolerance. Thus, Takeda et al. (18) suggest that Alzheimer amyloid pathology could aggravate diabetes.

Of note, these mouse models of AD and T2D have some limitations. First, leptin signaling is completely ablated because leptin production has been eliminated. Leptin signaling plays an important role in regulating synaptic function and cerebral energy homeostasis, and thus some of the phenotypes in APP+/−ob/ob mice might result from the lack of leptin signaling. Second, the ob/ob mice are obese, glucose intolerant, and insulin resistant at an early stage of development. Insulin signaling plays an important role regulating neuronal and synaptic functions (2, 20) in addition to regulating glucose homeostasis. Again, some of the metabolic phenotypes in those mice might result from impaired insulin signaling rather than APP23 overexpression. In contrast, our mouse model exhibits only partial leptin signaling deficiency in the absence of perturbed insulin signaling. Furthermore, db/+ mice do not exhibit obesity, glucose intolerance, or insulin resistance, avoiding interference to investigate the impact of aberrant Aβ production on glucose homeostasis.

Takeda et al. (18) used APP23 mice to reproduce Aβ deposition observed in AD patients. APP23 mice bear the Swedish mutation APP751, and the first deposits appear at 6 mo of age. However, severe Aβ deposition is observed only at later stages (~18 mo of age) (17). More recent models, including double- and triple-transgenic mice, have been developed to induce Aβ deposition at earlier ages, leading to a more severe version of the illness. In this sense, APPswe/PS1dE mice harboring the Swedish (K594M/N595L) mutation and exon-9-deleted PS1 (9) show Aβ deposition as early as 4 mo of age. By 6 mo of age these animals present robust senile plaque deposition in cortex and hippocampus (5), significantly shortening the time needed to reproduce AD-related pathology.

Studies in humans and mice indicate that T2D can predispose to dementia and AD. However, whether AD may affect the pathology of T2D remains incompletely understood (1, 10–12, 14, 15, 19). In this study, we showed that APPswe/PS1dE9 coexpression in the brain prompted the onset of glucose intolerance and insulin resistance accompanied by augmented pancreatic β-cell mass. Thus, in addition to the inability of insulin to repress the expression of hepatic PEPCK and G-6-Pase, it is plausible to hypothesize that aberrant plasma glucagon and/or glucocorticoid levels may also be contributing factors to explain the upregulation of PEPCK and G-6-Pase genes. Further work is warranted to investigate whether plasma Aβ might regulate the levels of glucagon and/or glucocorticoids.

It has been shown that insulin signaling is disrupted by soluble Aβ oligomers in cultures of hippocampal neurons (21). Thus, it is tempting to hypothesize that Aβ-mediated insulin
resistance in brain is one potential mechanism underlying the pathological alterations observed in patients with AD and T2D. This hypothesis is strengthened by the findings of Obici et al. (13), which demonstrated that disruption of hypothalamic insulin signaling impaired the ability of circulating insulin to inhibit glucose production, suggesting that hypothalamic insulin resistance can contribute to hyperglycemia in T2D. Interestingly, in our mouse model, Aβ overproduction in the brain (APPswe/PS1dE9 gene coexpression under the control of a prion promoter) results in elevated plasma Aβ levels. Therefore, peripheral Aβ might impair insulin signaling and contribute to postprandial glucose excursions and T2D. This notion is supported by two observations. First, there were no amyloid plaques at 7 wk of age, and second, we have demonstrated that high plasma Aβ levels were associated with insulin resistance in peripheral tissues such as skeletal muscle and liver. Nonetheless, we cannot exclude the hypothesis that Aβ mediates hypothalamic insulin resistance in our mouse model. Further studies are warranted to test this hypothesis.

In conclusion, we have generated a new mouse model to investigate the impact of Aβ on the early onset of T2D. We demonstrated that AD prompts the early onset of impaired insulin signaling and glucose intolerance. In addition, our findings support the notion that Aβ is a mechanistic link underlying AD and T2D and suggest that Aβ is a therapeutic target in the prevention and treatment of insulin resistance in AD patients.

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DISCLOSURES

The authors declare no conflicts of interest, financial or otherwise.

AUTHOR CONTRIBUTIONS


REFERENCES


