Increased Vulnerability of Brain Mitochondria in Diabetic (Goto-Kakizaki) Rats With Aging and Amyloid-β Exposure

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This study evaluated the respiratory indexes (respiratory control ratio [RCR] and ADP/O ratio), mitochondrial transmembrane potential (ΔΨm), repolarization lag phase, repolarization level, ATP/ADP ratio, and induction of the permeability transition pore of brain mitochondria isolated from normal Wistar and GK diabetic rats of different ages (1.5, 12, and 24 months of age). The effect of amyloid β-peptides, 50 μmol/l $A\beta_{25-35}$ or 2 μmol/l $A\beta_{1-40}$, on mitochondrial function was also analyzed. Aging of diabetic mice induced a decrease in brain mitochondrial RCR, ADP/O, and ATP/ADP ratios but induced an increase in the repolarization lag phase. Brain mitochondria from older diabetic rats were more prone to the induction of the permeability transition pore, i.e., mitochondria from 24-month-old diabetic rats accumulated much less Ca^{2+} (20 μmol/l) than those isolated from 12-month-old rats (50 μmol/l) or 1.5-month-old rats (100 μmol/l). In the presence of 50 μmol/l $A\beta_{25-35}$ or 2 μmol/l $A\beta_{1-40}$, age-related mitochondrial effects were potentiated. These results indicate that diabetes-related mitochondrial dysfunction is exacerbated by aging and/or by the presence of neurotoxic agents such as amyloid β-peptides, supporting the idea that diabetes and aging are risk factors for the neurodegeneration induced by these peptides. *Diabetes* 52: 1449–1456, 2003

Type 2 diabetes accounts for ~90% of the existing cases of diabetes and is characterized by defects in both insulin action and secretion (1). Many studies demonstrated that diabetes produces molecular, cellular, morphological, and behavioral changes in the central nervous system (CNS) (2). Pardridge et al (3) showed the existence of insulin receptors in the endothelium of the human blood-brain barrier, allowing receptor-mediated active transport of insulin into the brain. Insulin-sensitive glucose transporters are found not only at the blood-brain barrier but also in glia expressing partially insulin-sensitive GLUT1 (4,5) and in some neurons expressing GLUT1 (4) and/or GLUT4 (6,7). Recently, Bingham et al. (8) showed that insulin has a significant effect on global brain glucose metabolism, mainly in the cerebral cortex. The authors suggest that this effect may be either a direct effect of insulin, stimulating glucose uptake and metabolism, or an indirect effect achieved via insulin-stimulated neuronal activation with secondary increment in cell glucose metabolism. These results raise the hypothesis that insulin can access the insulin receptors in the brain and have a metabolic effect in this organ, which may be maximal at basal circulating insulin concentrations.

Diabetes is often associated with mitochondrial diseases characterized by defects in the mitochondrial genome (9). Mitochondria play a central role in the development of type 2 diabetes by regulating energy balance and the generation of reactive oxygen species (10). In Alzheimer's disease (AD), a major imbalance between glucose and oxygen consumption has been found in the incipient stage, whereas in the advanced stage, both glucose and oxygen consumption are diminished (11). This could be a consequence of deviant insulin action or brain insulin receptor function, which can affect brain energy metabolism (11). These abnormalities in insulin metabolism may account for the pathological changes (formation of senile plaques and neurofibrillary tangles) found in AD (12,13). Several lines of evidence suggest that amyloid deposition in the brain contributes to neuronal degeneration in AD (14) just as amyloid formation in the pancreas is believed to contribute to β-cell loss in type 2 diabetes (15). Hoyer (16) argued that in late-onset AD, a disturbance in the control of neuronal glucose metabolism consequent to impaired insulin signaling strongly resembles the pathophysiology of type 2 diabetes in nonneural tissue. Two recent prospective studies have established that diabetes increases the risk of dementia in general and AD in particular (17,18).

Several animal models are available for experimental investigation on type 2 diabetes. One of those models is the Goto-Kakizaki (GK) rat: a nonobese, spontaneously diabetic animal (19) produced by selective breeding of Wistar rats and first characterized by Goto and Kakizaki (20).

The aim of this work was to analyze the impact of aging and/or the presence of amyloid β-peptides (Aβ) on the function of brain mitochondria isolated from diabetic GK

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ΔΨm, transmembrane potential; AD, Alzheimer's disease; CNS, central nervous system; CsA, cyclosporin A; HNE, 4-hydroxynonenal; OXPHOS, oxidative phosphorylation; PTP, permeability transition pore; RCR, respiratory control ratio; TPP^+, tetraphenylphosphonium.

TABLE 1
Characterization of Wistar control and GK rats

	Wistar control (1.5 months; $n = 8$)	GK (1.5 months; $n = 8$)	Wistar control (12 months; $n = 7$)	GK (12 months; $n = 7$)	Wistar control (24 months; $n = 5$)	GK (24 months; $n = 5$)
Glycemia (mg/ml)	111.4 ± 9.9	223.6 ± 27.1[c]	102.0 ± 4.2[e]	256.8 ± 13.5[a,g]	85.5 ± 6.5[e,i]	180.5 ± 44.1
HbA_{1C} (%)	6.3 ± 0.7	6.0 ± 0.3	5.2 ± 0.1	9.6 ± 0.5[a,d,g]	5.2 ± 0.4[f,h,i]	7.7 ± 0.3[b,e,h,j,k]
GHb (%)	7.2 ± 0.9	6.6 ± 0.5	6.1 ± 0.1	12.6 ± 0.8[a,d,g]	5.9 ± 0.6[i]	9.8 ± 0.4[b,e,h,j,k]

Data are the means ± SE. HbA_{1c} and GHb data are expressed as a percentage of the total hemoglobin. [a]$P < 0.001$; [b]$P < 0.01$; [c]$P < 0.05$, when compared with mitochondria isolated from 1.5-month-old Wistar control rats. [d]$P < 0.001$, [e]$P < 0.01$; [f]$P < 0.05$, when compared with mitochondria isolated from 1.5-month-old GK rats. [g]$P < 0.001$; [h]$P < 0.01$, when compared with mitochondria isolated from 12-month-old Wistar control rats. [i]$P < 0.001$; [j]$P < 0.05$; when compared with mitochondria isolated from 12-month-old GK rats. [k]$P < 0.01$, when compared with mitochondria isolated from 24-month-old Wistar control rats.

rats. For this purpose, the following parameters were examined: mitochondrial respiration, mitochondrial transmembrane potential, levels of ATP, and the induction of the permeability transition pore. Studies on aging in an animal model of type 2 diabetes may identify a key metabolic feature common to that disorder and late-onset AD. One such feature could be a mitochondria-associated oxidative stress in AD brain (21,22) and in the brain of GK rats, as demonstrated previously in our laboratory (23).

RESEARCH DESIGN AND METHODS

Materials. $A\beta_{25-35}$ and $A\beta_{1-40}$ were obtained from Bachem AG (Bubendorf, Germany). Protease (Subtilisin Carlsberg) type VIII was obtained from Sigma (Sintra, Portugal). Digitonin was obtained from Calbiochem. All the other chemicals were of the highest grade of purity commercially available.

Animals. Male GK and control Wistar rats that were 1.5, 12, and 24 months of age were housed in our animal colony (Laboratory Research Center, University Hospital, Coimbra, Portugal). They were maintained under controlled light and humidity with free access to water and powdered rodent diet (diet C.R.F. 20, Charles River, L'Arbresle, France). Glucose tolerance tests were used to select GK rats for study. Adhering to procedures approved by the Institutional Animal Care and Use Committee, the animals were killed by cervical displacement and decapitation.

Determination of blood glucose and glycated hemoglobin (HbA_{1c}, GHb) levels. Immediately after the animals were killed, blood glucose was determined by a glucose oxidase reaction, using a glucometer and compatible reactive tests. GHb and HbA_{1c} levels were determined through ionic change chromatographic assay. GHb is a common term for posttranslationally modified molecules of HbA, resulting from a nonenzymatic binding of glucose (glycation) to the amino acid residues in α- and/or β-globin chains. Approximately 80% of GHb is HbA_{1c}, which is hemoglobin irreversibly glycated at one or both NH_2-terminal valine amino acid of each β-chain.

Isolation of brain mitochondria. Brain mitochondria were isolated from male Wistar and GK rats by the method of Rosenthal et al. (24), with slight modifications, using 0.02% digitonin to free mitochondria from the synaptosomal fraction. In brief, a rat was decapitated, and the whole brain minus the cerebellum was rapidly removed, washed, minced, and homogenized at 4°C in 10 ml of isolation medium (225 mmol/l mannitol, 75 mmol/l sucrose, 5 mmol/l HEPES, 1 mmol/l EGTA, 1 mg/ml BSA [pH 7.4]) containing 5 mg of the bacterial protease. Single brain homogenates were brought to 30 ml and then centrifuged at 2,000*g* for 3 min. The pellet, including the fluffy synaptosomal layer, was resuspended in 10 ml of the isolation medium containing 0.02% digitonin and centrifuged at 12,000*g* for 8 min. The brown mitochondrial pellet without the synaptosomal layer was then resuspended again in 10 ml of medium and recentrifuged at 12,000*g* for 10 min. The mitochondrial pellet was resuspended in 300 μl of resuspension medium (225 mmol/l mannitol, 75 mmol/l sucrose, 5 mmol/l HEPES [pH 7.4]). Mitochondrial protein was determined by the biuret method calibrated with BSA (25).

Membrane potential measurements. The mitochondrial transmembrane potential (ΔΨm) was monitored by evaluating the transmembrane distribution of tetraphenylphosphonium (TPP^+) with a TPP^+-selective electrode prepared according to Kamo et al. (26) using an $Ag/AgCl_2$ electrode as reference.

Reactions were carried out in a chamber with magnetic stirring in 1 ml of reaction medium (100 mmol/l sucrose, 100 mmol/l KCl, 2 mmol/l KH_2PO_4, 5 mmol/l HEPES, 10 μmol/l EGTA [pH 7.4]) supplemented with 3 μmol/l TPP^+. The experiments were started by adding 5 mmol/l succinate to mitochondria in suspension at 0.8 mg protein/ml. After a steady-state distribution of TPP^+ had been reached (ca. 2 min of recording), Ca^{2+} was added and ΔΨm was recorded. ΔΨm was estimated from the decrease of TPP^+ concentration in the reaction medium as described elsewhere (27). Homogenates were incubated with 50 μmol/l $A\beta_{25-35}$ or 2 μmol/l $A\beta_{1-40}$ for 5 min before succinate addition.

Mitochondrial respiration. Oxygen consumption of isolated mitochondria was monitored polarographically with a Clark oxygen electrode (28) connected to a suitable recorder in a 1-ml, thermostated, water-jacketed closed chamber, with magnetic stirring. The reactions were carried out at 30°C in 1 ml of the reaction medium with 0.8 mg of protein. Homogenates were incubated with 50 μmol/l $A\beta_{25-35}$ or 2 μmol/l $A\beta_{1-40}$ for 5 min before succinate addition.

Analysis of ATP. At the end of the ΔΨm experiments, each mitochondrial suspension was rapidly centrifuged at 14,000 rpm for 6 min with 0.3 mol/l perchloric acid. The supernatants were neutralized with 10 mol/l KOH in 5 mol/l Tris and centrifuged at 14,000 rpm for 5 min. The resulting supernatants were assayed for ATP by separation in a reverse-phase high-performance liquid chromatography. The chromatography apparatus was a Beckman-System Gold, consisting of a 126 Binary Pump Model and 166 Variable UV detector controlled by a computer. The detection wavelength was 254 nm, and the column was a Lichrosphere 100 RP-18 (5 μm) from Merck. An isocratic elution with 100 mmol/l phosphate buffer (KH_2PO_4; pH 6.5) and 1.0% methanol was performed with a flow rate of 1 ml/min. The required time for each analysis was 6 min.

Statistical analysis. Results are presented as mean ± SE of the indicated number of experiments. Statistical significance was determined using the one-way ANOVA test for multiple comparisons, followed by the post hoc Tukey-Kramer test. $P < 0.05$ was considered significant.

RESULTS

Glycemia and HbA_{1c} levels in Wistar control and GK rats. For confirming diabetes in GK rats, glycemia and the glycated hemoglobin (HbA_{1c}, GHb) levels were determined (Table 1). The percentage of hemoglobin in glycated form (GHb and HbA_{1C}) was significantly higher in GK than in Wistar control rats (Table 1). Similarly, blood glucose levels increased in GK rats when compared with Wistar control rats. However, the glycemia of 12-month-old GK rats was higher than that of 24-month-old rats.

Effect of aging and Aβ on brain mitochondrial respiration. Respiratory control ratio (RCR) is the ratio between mitochondrial respiration states 3 (consumption of oxygen in the presence of substrate and ADP) and 4 (consumption of oxygen after ADP has been consumed). RCR decreased with aging (Fig. 1*A*). The ADP/O ratio also showed a significant decrease with age (Fig. 2*A*). The ADP/O ratio, an indicator of oxidative phosphorylation efficiency, is expressed by the ratio between the amount of ADP added and the oxygen consumed during state 3 respiration. In the presence of 50 μmol/l $A\beta_{25-35}$ or 2 μmol/l $A\beta_{1-40}$, the effects of age on both respiratory indexes were exacerbated: the decreases on RCR (Fig. 1*A*) and ADP/O ratio (Fig. 2*A*) were potentiated. A similar pattern occurred in Wistar control rats (Fig. 1*B* and 2*B*).

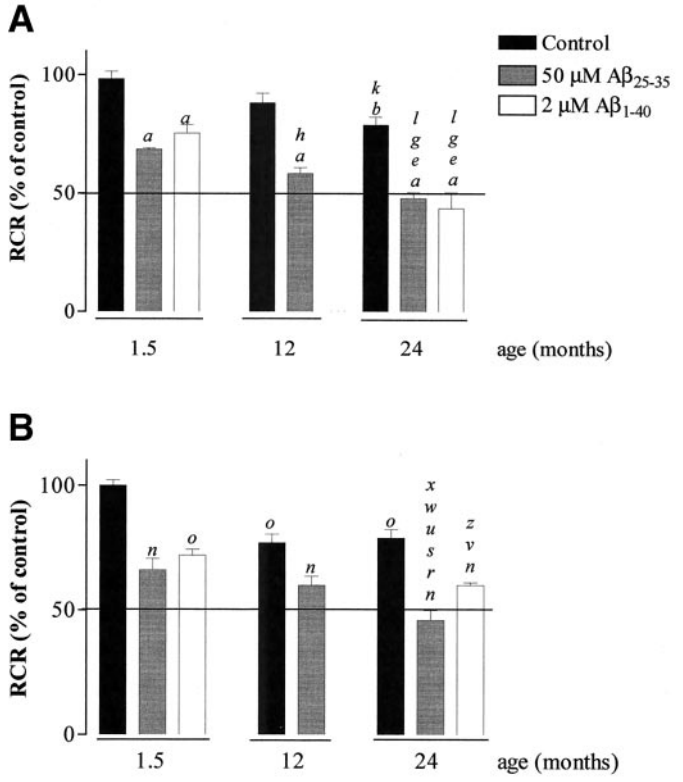

FIG. 1. Effect of age and Aβ peptides on RCR of brain mitochondria isolated from GK (*A*) and Wistar control (*B*) rats. Freshly isolated brain mitochondria (0.8 mg) in 1 ml of the standard medium supplemented with 2 μmol/l rotenone were energized with 5 mmol/l succinate. Isolates were preincubated with 50 μmol/l $A\beta_{25-35}$ and 2 μmol/l $A\beta_{1-40}$ for 5 min at 30°C before mitochondria energization. $^{a}P < 0.001$; $^{b}P < 0.01$, when compared with mitochondria isolated from 1.5-month-old GK rats. $^{e}P < 0.01$, when compared with mitochondria isolated from 1.5-month-old GK rats, in the presence of 2 μmol/l $A\beta_{1-40}$. $^{g}P < 0.001$; $^{h}P < 0.01$, when compared with mitochondria isolated from 12-month-old GK rats. $^{k}P < 0.05$, when compared with mitochondria isolated from 12-month-old GK rats, in the presence of 50 μmol/l $A\beta_{25-35}$. $^{l}P < 0.001$, when compared with mitochondria isolated from 24-month-old GK rats. $^{n}P < 0.001$, when compared with mitochondria isolated from 1.5-month-old Wistar control rats. $^{r}P < 0.05$, when compared with mitochondria isolated from 1.5-month-old Wistar control rats, in the presence of 50 μmol/l $A\beta_{25-35}$. $^{s}P < 0.001$, when compared with mitochondria isolated from 1.5-month-old Wistar control rats, in the presence of 2 μmol/l $A\beta_{1-40}$. $^{u}P < 0.001$; $^{v}P < 0.05$, when compared with mitochondria isolated from 12-month-old Wistar control rats. $^{w}P < 0.05$, when compared with mitochondria isolated from 12-month-old Wistar control rats, in the presence of 50 μmol/l $A\beta_{25-35}$. $^{x}P < 0.001$, when compared with mitochondria isolated from 24-month-old Wistar control rats.

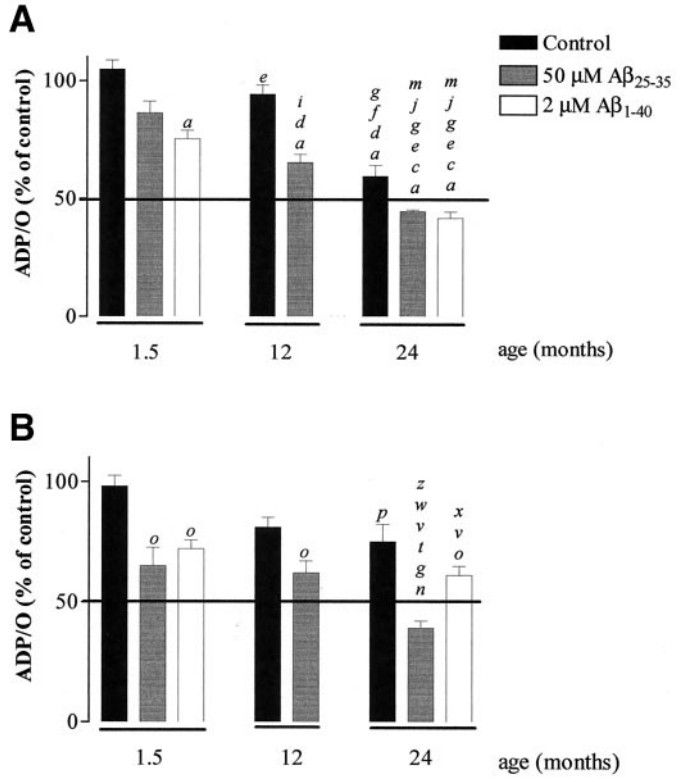

FIG. 2. Effect of aging and Aβ peptides on ADP/O ratio of brain mitochondria isolated from GK (*A*) and Wistar control (*B*) rats. Freshly isolated brain mitochondria (0.8 mg) in 1 ml of the standard medium supplemented with 2 μmol/l rotenone were energized with 5 mmol/l succinate. Isolates were preincubated with 50 μmol/l $A\beta_{25-35}$ and 2 μmol/l $A\beta_{1-40}$ for 5 min at 30°C before mitochondria energization. $^{a}P < 0.001$, when compared with mitochondria isolated from 1.5-month-old GK rats. $^{c}P < 0.001$; $^{d}P < 0.01$, when compared with mitochondria isolated from 1.5-month-old GK rats, in the presence of $A\beta_{25-35}$. $^{e}P < 0.01$; $^{f}P < 0.05$, when compared with mitochondria isolated from 1.5-month-old GK rats, in the presence of $A\beta_{1-40}$. $^{g}P < 0.001$; $^{i}P < 0.05$, when compared with mitochondria isolated from 12-month-old GK rats. $^{n}P < 0.001$; $^{o}P < 0.01$; $^{p}P < 0.05$, when compared with mitochondria isolated from 1.5-month-old Wistar control rats. $^{q}P < 0.01$, when compared with mitochondria isolated from 1.5-month-old Wistar control rats, in the presence of $A\beta_{25-35}$. $^{t}P < 0.01$, when compared with mitochondria isolated from 1.5-month-old Wistar control rats, in the presence of $A\beta_{1-40}$. $^{v}P < 0.05$, when compared with mitochondria isolated from 12-month-old Wistar control rats. $^{y}P < 0.01$, when compared with mitochondria isolated from 24-month-old Wistar control rats.

However, the effects of aging and Aβ peptides were less pronounced than those observed in 12- and 24-month-old GK rats.

Effect of aging and Aβ on ΔΨm, repolarization lag phase, repolarization level, and ATP/ADP ratio. The mitochondrial ΔΨm is fundamental for the phenomenon of oxidative phosphorylation, the conversion of ADP to ATP via ATP synthase. Mitochondrial respiratory chain pumps H^+ out of the mitochondrial matrix across the inner mitochondrial membrane. The H^+ gradient establishes an electrochemical potential (Δp) resulting in a pH (ΔpH) and a voltage gradient (ΔΨm) across the mitochondrial inner membrane. Figure 3 is a representative trace of the effect of $A\beta_{25-35}$ on ΔΨm (decreased), lag phase (increased), and repolarization phase (decreased) of mitochondria isolated from 1.5-month-old diabetic rats. As shown in Table 2, age did not affect substantially the ΔΨm. However, the presence of 50 μmol/l $A\beta_{25-35}$ or 2 μmol/l $A\beta_{1-40}$ led to a significant decrease of ΔΨm in mitochondria isolated from 24-month-old rats (Table 2).

Similarly, aging did not affect either repolarization lag phase (corresponding to ADP phosphorylation) or repolarization level (time necessary for mitochondria to reestablish the ΔΨm, after ADP phosphorylation) of diabetic brain mitochondria (Table 2). However, the presence of 50 μmol/l $A\beta_{25-35}$ or 2 μmol/l $A\beta_{1-40}$ led to a significant decrease of both parameters. In the presence of 2 μmol/l $A\beta_{1-40}$, those parameters could not be evaluated in mitochondria isolated from 24-month-old rats because after depolarization induced by ADP, the basal ΔΨm could not be reestablished as a result of the inability of mitochondria to phosphorylate all of the ADP added, in the presence of the peptide.

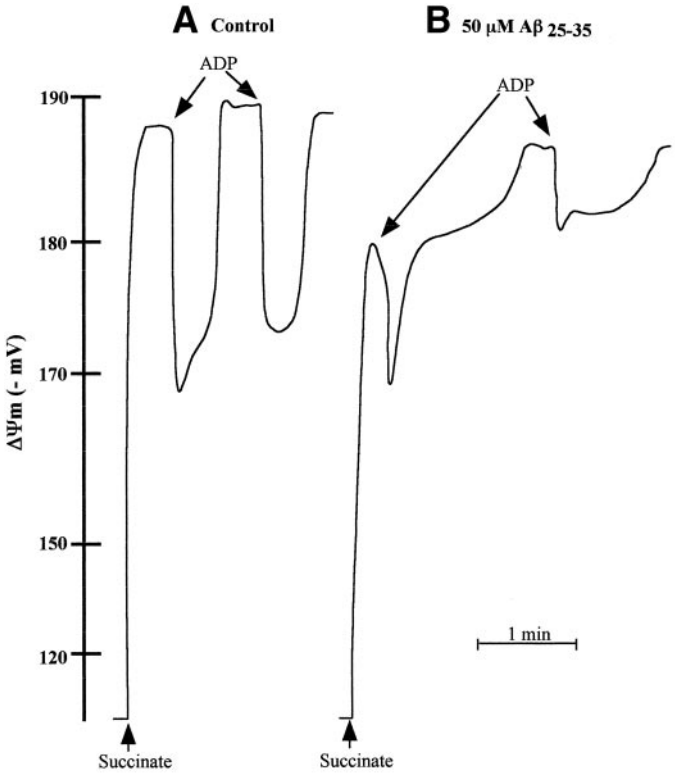

FIG. 3. Representative trace of the effect of $A\beta_{25-35}$ on ΔΨm, repolarization lag phase, and repolarization level of mitochondria isolated from 24-month-old GK rats. Freshly isolated brain mitochondria (0.8 mg) in 1 ml of the standard medium supplemented with 3 μmol/l TPP^+ and 2 μmol/l rotenone were energized with 5 mmol/l succinate. Isolates were preincubated with 50 μmol/l $A\beta_{25-35}$ for 5 min at 30°C before mitochondria energization.

As shown in Fig. 4*A*, an age-related decrease in ATP/ADP ratio of diabetic brain mitochondria was observed. The presence of 50 μmol/l $A\beta_{25-35}$ or 2 μmol/l $A\beta_{1-40}$ exacerbated the decrease on ATP levels, this effect being more pronounced in the presence of 2 μmol/l $A\beta_{1-40}$. A similar pattern occurred with Wistar control rats (Fig. 4*B*). However, the effects of aging and Aβ were less pronounced than those observed in 12- and 24-month-old GK rats.

Effect of aging and Aβ on the induction of mitochondrial permeability transition pore. The drop of ΔΨm is a typical phenomenon that leads to the induction of permeability transition pore (PTP). Figures 5 and 6 show the alteration of brain mitochondrial electric potential (ΔΨm) induced by age and $A\beta_{25-35}$, respectively.

In mitochondria isolated from 1.5-month-old GK rats, after energizing with succinate, the first pulse of 50 μmol/l Ca^{2+} led to a rapid depolarization followed by a partial repolarization. However, a second pulse of Ca^{2+} led to an irreversible depolarization (Fig. 5*A*).

Mitochondria isolated from 12- and 24-month-old GK rats showed a smaller capacity to accumulate Ca^{2+}. They undergo PTP induction after two pulses of 25 μmol/l Ca^{2+} or 10 μmol/l Ca^{2+}, respectively (Fig. 5*B* and 5*C*). The collapse of ΔΨm was prevented by adding EGTA or oligomycin plus ADP, which completely restored ΔΨm to the state 4 level (e.g., before Ca^{2+} addition) (Fig. 5*B*). In mitochondria isolated from 24-month-old rats, $A\beta_{25-35}$ (50 μmol/l; Fig. 6) induced a significant decrease of ΔΨm measured after mitochondria energization. These mitochondria were more susceptible to the amount of Ca^{2+} added, because they undergo PTP in the presence of lower Ca^{2+} concentrations. The higher susceptibility to Ca^{2+} addition induced by $A\beta_{25-35}$ also occurred in mitochondria isolated from 1.5- and 12-month-old GK rats (data not shown). The presence of 0.85 μmol/l cyclosporin A (CsA; specific inhibitor of PTP) added 2 min before Ca^{2+} afforded a clear protection of mitochondria because it prevents the depolarization induced by Ca^{2+} (Fig. 7*A*). In addition, the preincubation (2 min) of mitochondria with 1 mmol/l ADP plus 2 μg/ml oligomycin completely prevented mitochondria depolarization by increasing dramatically the repolarization capacity of mitochondrial membrane after Ca^{2+} accumulation (Fig. 7*B*).

DISCUSSION

The effect of Aβ on the function of brain mitochondria was analyzed in GK rats at different ages (1.5, 12, and 24 months). We observed that aging renders diabetic brain mitochondria more susceptible to toxic insults such as the neurotoxic Aβ.

The characterization of diabetes in GK rats was performed by determining the blood levels of glucose and glycated hemoglobin (HbA_{1C} and GHb). A significant increase in GHb and also in glycemia was observed (Table 1). We should note the decrease, although not significant, in glycemia of 24-month-old GK rats, which is associated with weight loss, i.e., 24-month-old GK rats presented a loss in body weight, which may be responsible for glycemia decrement. Besides the genetic predisposition, obesity is the most important risk factor for the development of type 2 diabetes. Data from the literature indicate that weight loss can result in a significant improvement in blood glucose levels (29).

The isolation procedure used in this study does not allow separation of mitochondria from different cell types. Consequently, the observed alterations in mitochondrial function may derive from changes in one or more cell types. They include the three types of insulin-sensitive cells in the brain: vascular epithelial cells, astrocytes, and some neurons. All three of these cell types are affected in diabetes. Of great interest here, however, are astrocytes and neurons, because mitochondria in these cell types are known to suffer morphological changes in diabetic rats (30,31). Astrocytes, which are intimately involved in neuronal function, play an important role in brain glucose metabolism (32) and thus merit as much attention as neurons in subsequent studies of brain mitochondrial changes in GK rats.

Hyperglycemia in GK rats could cause brain mitochondrial impairment via oxidative stress compromising brain function. Data from the literature show that hyperglycemia induces oxidative damage in rat brain (23,33). It was also shown that lipid peroxidation products are increased in the brain of type 2 diabetic mice, whereas the activity of antioxidant enzymes, such as catalase and superoxide dismutase, is decreased (34,35). The presence of lipid peroxidation products, such as 4-hydroxynonenal (HNE), reduces the activity of a variety of enzymes that are critical to normal function of neurons, including glucose transporters (36). HNE has been demonstrated to have the

FIG. 4. Effect of age and Aβ peptides on ATP/ADP ratio of brain mitochondria isolated from GK (*A*) and Wistar control (*B*) rats. At the end of incubations such as those described in Figs. 1, 2, and 3, mitochondrial suspensions were centrifuged and processed for ATP levels analysis as described in RESEARCH DESIGN AND METHODS. Isolates were preincubated with 50 µmol/l $A\beta_{25-35}$ and 2 µmol/l $A\beta_{1-40}$ for 5 min at 30°C before mitochondria energization. $^{a}P < 0.001$; $^{b}P < 0.05$, when compared with mitochondria isolated from 1.5-month-old GK rats; $^{c}P < 0.01$, $^{d}P < 0.05$, when compared with mitochondria isolated from rats 1.5-month-old GK rats, in the presence of $A\beta_{25-35}$; $^{e}P < 0.01$, when compared with mitochondria isolated from rats 1.5-month-old GK rats, in the presence of $A\beta_{1-40}$; $^{f}P < 0.05$, when compared with mitochondria isolated from rats 12-month-old GK rats. $^{g}P < 0.001$; $^{h}P < 0.01$, when compared with mitochondria isolated from 1.5-month-old Wistar control rats; $^{i}P < 0.001$; $^{j}P < 0.01$, when compared with mitochondria isolated from 1.5-month-old Wistar control rats, in the presence of $A\beta_{25-35}$; $^{k}P < 0.001$, when compared with mitochondria isolated from 1.5-month-old GK rats, in the presence of $A\beta_{1-40}$; $^{l}P < 0.001$; $^{m}P < 0.01$, when compared with mitochondria isolated from 24-month-old Wistar control rats.

TABLE 2
Effect of age and Aβ on mitochondrial ΔΨm, repolarization lag phase, and repolarization level

	1.5 months ($n = 8$)	1.5 months + 50 µmol/l $A\beta_{25-35}$ ($n = 8$)	1.5 months + 2 µmol/l $A\beta_{1-40}$ ($n = 8$)	12 months ($n = 6$)	12 months + 50 µmol/l $A\beta_{25-35}$ ($n = 6$)	24 months ($n = 5$)	24 months + 50 µmol/l $A\beta_{25-35}$ ($n = 5$)	24 months + 2 µmol/l $A\beta_{1-40}$ ($n = 5$)
ΔΨm	100.4% ± 0.6	98.24% ± 1.1	98.03% ± 0.6	99.11% ± 1.3	99.03% ± 0.6	99.12% ± 0.9	93.35% ± 1.6^{a,d,g,j,m,o}	94.5% ± 0.8^{a,d,g,j,m,o}
Repolarization lag phase	93.16% ± 3.2	102.7% ± 8.3^{b}	131.6% ± 6^{a}	102.7% ± 13.1	171.9% ± 20^{a,c,g,k}	111.8% ± 4.3^{e,h,l}	233.4% ± 27.3^{a,c,f,i,n,o}	n.d.
Repolarization level	109.9% ± 7.3	81% ± 7.2^{b}	70.4% ± 2.7^{a}	100.8% ± 6.2^{e,g}	62.8% ± 2.5^{a,c,h}	105.3% ± 4.9^{e,g,l}	48.5% ± 5.5^{a,d,g,i,m,o}	n.d.

Freshly isolated brain mitochondria (0.8 mg) in 1 ml of the reaction medium supplemented with 3 µmol/l TPP^{+} and 2 µmol/l rotenone were energized with 5 mmol/l succinate. Homogenates were preincubated with 50 µmol/l $A\beta_{35-25}$ or 2 µmol/l $A\beta_{1-40}$ for 5 min at 30 °C before mitochondria energization. $^{a}P < 0.001$; $^{b}P < 0.01$, when compared with mitochondria isolated from 1.5-month-old GK rats. $^{c}P < 0.001$; $^{d}P < 0.01$; $^{e}P < 0.05$, when compared with mitochondria isolated from 1.5-month-old GK rats and in the presence of 50 µmol/l $A\beta_{25-35}$; $^{f}P < 0.001$; $^{g}P < 0.01$; $^{h}P < 0.05$, when compared with mitochondria isolated from 1.5-month-old GK rats and in the presence of 2 µmol/l $A\beta_{1-40}$; $^{i}P < 0.001$; $^{j}P < 0.01$; $^{k}P < 0.05$, when compared with mitochondria isolated from 12-month-old GK rats. $^{l}P < 0.001$; $^{m}P < 0.01$; $^{n}P < 0.05$, when compared with mitochondria isolated from 12-month-old GK rats and in the presence of 50 µmol/l $A\beta_{25-35}$. $^{o}P < 0.001$, when compared with mitochondria isolated from 24-month-old GK rats.

capacity to impair mitochondrial function in brain synaptosomes (36). The specific mitochondrial enzymes α-ketoglutarate dehydrogenase and pyruvate dehydrogenase have been shown to be targets for HNE protein conjugation, resulting in a decrease of the activity of both enzymes (37).

Dysfunction of the mitochondrial respiratory chain has been described in diabetes (38). Accordingly, our data show an age-related impairment of the respiratory chain (decrease of RCR; Fig. 1*A*) and an uncoupling of oxidative phosphorylation (OXPHOS; decrease of ADP/O ratio; Fig. 2*A*) in brain mitochondria isolated from GK rats. Inhibition of cellular energy production has been shown to reduce or abolish both insulin secretion and action (39). In addition, the decrement in OXPHOS efficiency is related to a loss in the control of glucose homeostasis as evidenced by the increase in tissue and blood lactate levels, as well as by the change in glucose tolerance (Table 1). The impairment of

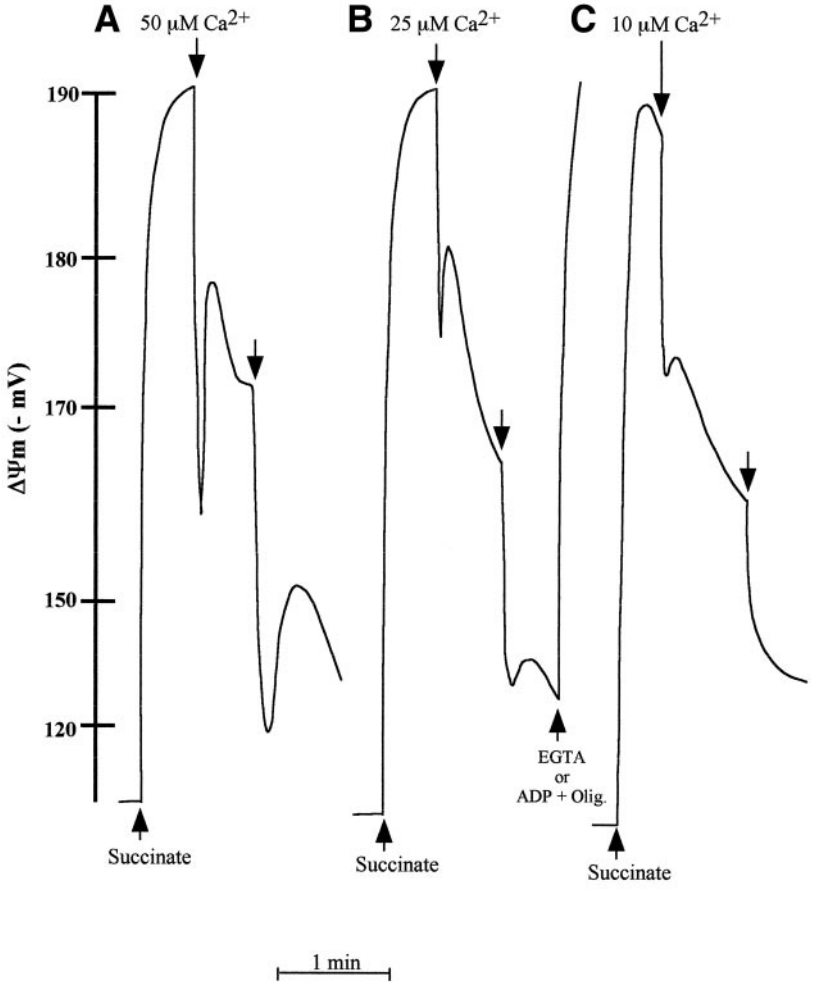

FIG. 5. Effect of age on PTP induction: susceptibility to Ca^{2+} addition. Freshly isolated brain mitochondria (0.8 mg) in 1 ml of the standard medium supplemented with 3 μmol/l TPP^+ and 2 μmol/l rotenone were energized with 5 mmol/l succinate. *A*: Mitochondria isolated from 1.5-month-old GK rats. *B*: Mitochondria isolated from 12-month-old GK rats. *C*: Mitochondria isolated from 24-month-old GK rats. Ca^{2+} was added 1.5 min after mitochondria energization. The traces are typical of three experiments.

the respiratory chain (Fig. 1*A*) and the uncoupling of OXPHOS (Fig. 2*A*) are not accompanied by a decrease in ΔΨm (Table 2). However, an age-related decrease in ATP/ADP ratio of mitochondria isolated from diabetic rats occurred (Fig. 4*A*). Cybrid cells constructed from individuals with maternally inherited diabetes exhibited lactic acidosis, poor respiration, and marked defects in mitochondrial morphology and respiratory chain complex I and IV activities (40). Because CNS depends so heavily on ATP production, the inhibition of OXPHOS will affect this system before any other system. For example, CNS requires a large amount of ATP for the transmission of impulses along the neural pathway; thus, mitochondrial function impairment will result in neurodegeneration and loss in neuronal metabolic control (41,42).

Bioenergetic complications induced by diabetes are exacerbated by aging. Our data show a more pronounced mitochondrial dysfunction in older GK rats (12 and 24 months old) (Figs. 1*A*, 2*A*, and 4*A*) when compared with Wistar control rats of the same age (Figs. 1*B*, 2*B*, and 4*B*). This indicates that aging is a risk factor for diabetes increasing the susceptibility to neurotoxic agents. Diabetes leads to functional and structural changes in the brain that seem to be most pronounced in the elderly. Increased age is associated with insulin resistance (43). Increasing data support the idea that mitochondrial function declines with aging and in age-related diseases such as diabetes and AD (41,42). Brain mitochondria of GK rats presented an age-related susceptibility to Ca^{2+}, indicating that aging predisposes diabetic rat mitochondria to the opening of the PTP (Fig. 5). PTP induction is a well-characterized process that results in a nonselective increase in the permeability of the inner mitochondrial membrane to solutes smaller than 1.5 kDa. Criterial attributes of the PTP include the dependence on matrix Ca^{2+} concentration and inhibition by the immunosuppressant CsA (44).

The maintenance of calcium homeostasis represents a major expenditure within neurons and, through respiratory control mechanisms, is tightly coupled to the rates of OXPHOS and the generation of reactive oxygen species. Recently, we demonstrated that Aβ and/or Ca^{2+} induce PTP opening of brain mitochondria (45,46). However, the opening of PTP may be avoided in the presence of CsA (specific inhibitor of PTP) and ADP plus oligomycin (Fig. 7), as previously described (47–49).

Another interesting feature is the effect of Aβ on brain bioenergetics of GK rats. Our results indicate that amyloid β-peptides exacerbate the effects of age-related diabetes. They potentiate respiratory chain impairment (Fig. 1), uncoupling of OXPHOS (Fig. 2), a decrease in ATP levels (Fig. 4), and the susceptibility to PTP opening (Fig. 6). Several comparisons can be made between the chronic effects of diabetes and the neurological impairments observed in AD. In patients with AD, an increase in cognitive

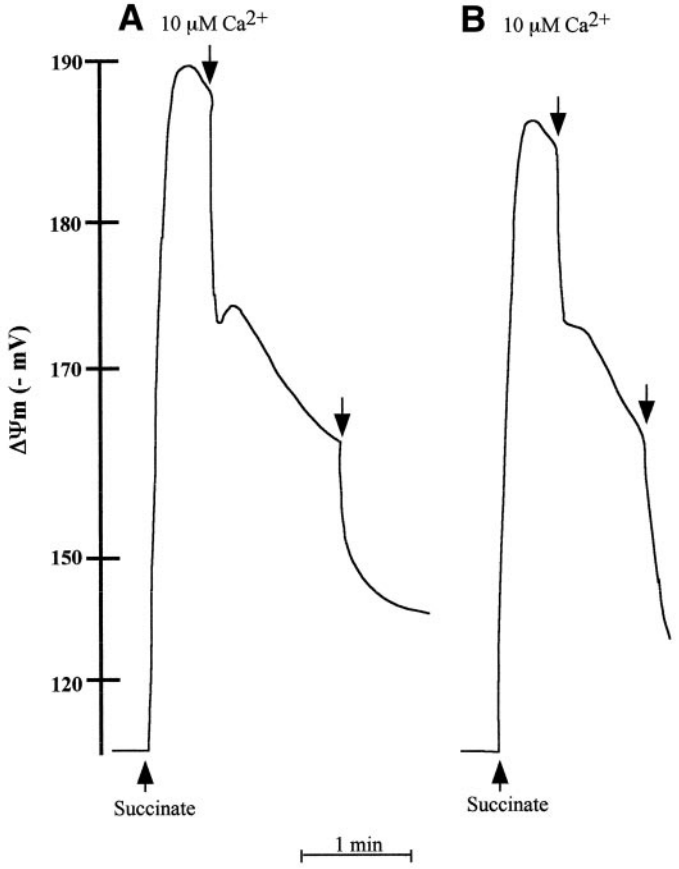

FIG. 6. Effect of $A\beta_{25-35}$ on $\Delta\Psi m$ of mitochondria isolated from 24-month-old GK rats. Freshly isolated brain mitochondria (0.8 mg) in 1 ml of the standard medium supplemented with 3 μmol/l TPP^+ and 2 μmol/l rotenone were energized with 5 mmol/l succinate. *A*: Control. *B*: 50 μmol/l $A\beta_{25-35}$ was preincubated for 5 min at 30°C before mitochondria energization. Ca^{2+} (100 μmol/l) was added 1 min after mitochondria energization. The traces are typical of three experiments.

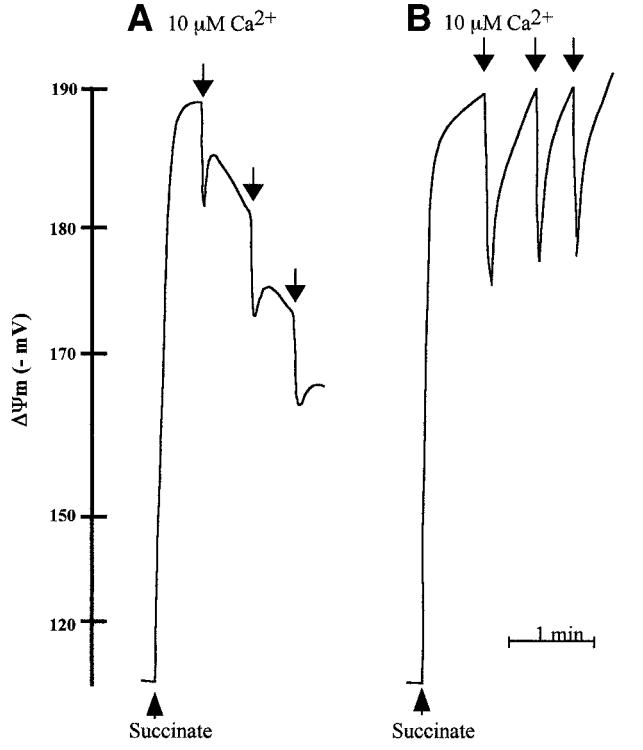

FIG. 7. Inhibitory effect of CsA (*A*) and oligomycin plus ADP (*B*) on Ca^{2+}-dependent PTP opening. Mitochondria were incubated in 0.8 mg protein/ml under standard conditions as described in RESEARCH DESIGN AND METHODS. A total of 0.85 μmol/l CsA and 1 mmol/l ADP plus 2 μg/ml oligomycin were incubated with mitochondria for 2 min before adding Ca^{2+}. The traces are typical of three experiments.

function in response to glucose administration and insulin therapy has been demonstrated (50), presumably as a result of an increase in hippocampal glucose utilization. Neuronal glucose transport and utilization have been shown to be reduced in AD (51). In streptozotocin-treated rats, a model of type 1 diabetes, β-amyloid toxicity is potentiated in the hippocampus (52). Furthermore, diabetes and AD have been shown to be associated with mitochondrial dysfunction. Both diseases occur with impaired glucose utilization and deficits in mitochondrial activity, and metabolic dysfunction is an important component in both diseases (38,41). The similarities between AD and the neurological consequences of diabetes raised the hypothesis that the life-long effects of hyperglycemia may predispose patients with diabetes to AD (53). In AD, the desensitization of the neuronal insulin receptor similar to what occurs in type 2 diabetes may be of pivotal significance. This abnormality, along with a reduction in brain insulin concentration, is assumed to induce several disturbances, including changes in cellular glucose, acetylcholine, cholesterol, and ATP, which are associated with abnormalities in cellular homeostasis and with the formation of both amyloidogenic derivatives and hyperphosphorylated tau protein (13).

Our results are consistent with the view that diabetes-related mitochondrial dysfunction is exacerbated by aging and/or by the presence of neurotoxic agents, such as Aβ, suggesting that diabetes and aging are risk factors for the neurodegeneration induced by these peptides. This study supports the idea, previously suggested by others (13,16), that a strong correlation exists between both age-related pathologies, diabetes and AD, mitochondria being a fundamental link in this process.

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REFERENCES

1. Gavin JR III, Alberti KGMM, Davidson MB, DeFronzo RA, Drash A, Gabbe SG, Genuth S, Harris MI, Kahn R, Keen H, Knowler WC, Lebovitz H, Maclaren NK, Palmer JP, Raskin P, Rizza RA, Stern MP: Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care* 20:1183–1197, 1997
2. Biessels GJ, van der Heide LP, Kamal A, Bleys RLAW, Gispen WH: Ageing and diabetes: implications for brain function. *Eur J Pharmacol* 441:1–14, 2002
3. Pardridge WM, Eisenberg J, Yang J: Human blood brain barrier insulin receptor. *J Neurochem* 44:1771–1778, 1985
4. Vannucci SJ, Maher F, Simpson IA: Glucose transporter proteins in brain: delivery of glucose to neurons and glia. *Glia* 21:2–21, 1997
5. Ngarmukos C, Baur EL, Kumagai AK: Co-localisation of GLUT1 and GLUT4 in the blood brain barrier of the ventromedial hypothalamus. *Brain Res* 900:1–8, 2001

6. Cheng CM, Cohen M, Wang J, Bondy CA: Estrogen augments glucose transporter and IGF1 expression in primate cerebral cortex. *FASEB J* 15:907–915, 2001
7. Choeiri C, Staines W, Messier C: Immunohistochemical localization and quantification of glucose transporters in the mouse brain. *Neuroscience* 111:19–34, 2002
8. Bingham EM, Hopkins D, Smith D, Pernet A, Hallett W, Reed L, Marsden PK, Amiel SA: The role of insulin in human brain glucose metabolism: an 18fluoro-deoxyglucose positron emission tomography study. *Diabetes* 51: 3384–3390, 2002
9. Gerbitz KD, van den Ouweland JM, Maassen JA, Jaksch M: Mitochondrial diabetes mellitus: a review. *Biochim Biophys Acta* 1271:253–260, 1995
10. Wallace DC: Mitochondrial diseases in man and mouse. *Science* 283:1482–1488, 1999
11. Hoyer S: Abnormalities of glucose metabolism in Alzheimer's disease. *Ann N Y Acad Sci* 640:53–58, 1991
12. Frölich L, Blum-Degen D, Bernstein HG, Engelsberger S, Humrich J, Laufer S, Muschner D, Thalheimer A, Turk A, Hoyer S, Zochling R, Boissl KW, Jellinger K, Riederer P: Brain insulin and insulin receptors in aging and sporadic Alzheimer's disease. *J Neural Transm* 105:423–438, 1998
13. Hoyer S: The aging brain. Changes in the neuronal insulin/insulin receptor signal transduction cascade trigger late-onset sporadic Alzheimer disease (SAD). *J Neural Transm* 109:991–1002, 2002
14. Selkoe DJ: Alzheimer's disease: genes, proteins, and therapy. *Physiol Rev* 81:741–766, 2001
15. Höppener JWM, Ahrén B, Lips CJM: Mechanisms of disease: islet amyloid and type 2 diabetes mellitus. *N Engl J Med* 343:411–419, 2000
16. Hoyer S: Risk factors for Alzheimer's disease during aging: impacts of glucose/energy metabolism. *J Neurol Transm* 54:187–194, 1998
17. Leibson CL, Rocca WA, Hanson VA, Cha R, Kokmen E, O'Brien PC, Palumbo PJ: Risk of dementia among persons with diabetes mellitus and the risk of dementia: a population-based cohort study. *Am J Epidemiol* 145:301–308, 1997
18. Ott A, Stolk RP, van Harskamp F, Pols HAP, Hofman A, Breteler MMB: Diabetes mellitus and the risk of dementia: the Rotterdam Study. *Neurology* 53:1937–1942, 1999
19. Serradas P, Gangnerau MN, Giroix MH, Saulnier C, Portha B: Impaired pancreatic beta cell function in the fetal GK rat: impact of diabetic inheritance. *J Clin Invest* 101:899–904, 1998
20. Goto Y, Kakizaki M: The spontaneous diabetic rat a model of non-insulin dependent diabetes mellitus. *Proc Jpn Acad* 57:381–384, 1981
21. Mark RJ, Pang Z, Geddes JW, Uchida K, Mattson MP: Amyloid beta-peptide impairs glucose transport in hippocampal and cortical neurons: involvement of membrane lipid peroxidation. *J Neurosci* 17:1046–1054, 1997
22. Bonilla E, Tanji K, Hirano M, Vu TH, DiMauro S, Schon EA: Mitochondrial involvement in Alzheimer's disease. *Biochim Biophys Acta* 1410:171–182, 1999
23. Santos MS, Santos DL, Palmeira CM, Seiça R, Moreno AJ, Oliveira CR: Brain and liver mitochondria isolated from diabetic Goto-Kakizaki rats show different susceptibility to induced oxidative stress. *Diabetes Metab Res Rev* 17:223–230, 2001
24. Rosenthal RE, Hamud F, Fiskum G, Varghese PJ, Sharpe S: Cerebral ischemia and reperfusion: prevention of brain mitochondrial injury by lidoflazine. *J Cereb Blood Flow Metab* 7:752–758, 1987
25. Gornall AG, Bardawill CJ, David MM: Determination of serum proteins by means of the biuret reaction. *J Biol Chem* 177:751–766, 1949
26. Kamo N, Muratsugu M, Hongoh R, Kobatake V: Membrane potential of mitochondria measured with an electrode sensitive to tetraphenylphosphonium and relationship between proton electrochemical potential and phosphorylation potential in steady state. *J Membr Biol* 49:105–121, 1979
27. Moreno AJM, Madeira VMC: Mitochondrial bioenergetics as affected by DTT. *Biochim Biophys Acta* 1060:166–174, 1991
28. Estabrook RE: Mitochondrial respiratory control and the polarographic measurement of ADP/O ratios. *Methods Enzymol* 10:41–47, 1967
29. Carapetis M, Phillips P: Eat less, walk more: enjoyable eating for type 2 diabetes. *Aust Fam Physician* 31:1065–1071, 2002
30. McCuskey PA, McCuskey RS: In vivo and electron microscopic study of the development of cerebral diabetic microangiography. *Microcirc Endothelium Lymphatics* 1:221–241, 1984
31. Piotrowski P, Gajkowska B, Olszewska H, Smialek M: Electron microscopy studies on experimental diabetes and cerebral ischemia in the rat brain. *Folia Neuropathol* 37:256–263, 1999
32. Forsyth RJ: Astrocytes and the delivery of glucose from plasma to neurons. *Neurochem Int* 28:231–241, 1996
33. Ceriello A, Giugliano D, Quatrapo A, Dello Russo P, Lefèbvre PJ: Metabolic control may influence the increased superoxide generation in diabetic serum. *Diabet Med* 8:540–542, 1991
34. Kumar JSS, Menon VP: Effect of diabetes on levels of lipid peroxides and glycolipids in rat brain. *Metabolism* 42:1435–1439, 1993
35. Makar TK, Hungund BL, Cook GA, Kashfi K, Cooper AJL: Lipid metabolism and membrane composition are altered in the brains of type II diabetic mice. *J Neurochem* 64:2159–2168, 1995
36. Mattson MP: Modification of ion homeostasis by lipid peroxidation: roles in neuronal degeneration and adaptative plasticity. *Trends Neurosci* 21:53–57, 1998
37. Humphries KM, Szweda LI: Selective inactivation of α-ketoglutarate dehydrogenase and pyruvate dehydrogenase: reaction of lipoic acid with 4-hydroxy-2-nonenal. *Biochemistry* 37:15835–15841, 1998
38. Kristal BS, Jackson CT, Chung HY, Matsuda M, Nguyen HD, Yu BP: Defects at center P underlie diabetes-associated mitochondrial dysfunction. *Free Radic Biol Med* 22:823–833, 1997
39. Gerbitz KD, Gempel K, Brdiczka D: Mitochondria and diabetes: genetic, biochemical, and clinical implications of the cellular energy circuit. *Diabetes* 45:113–126, 1996
40. van den Ouweland JM, Maechler P, Wollheim CB, Attardi G, Maassen JA: Functional and morphological abnormalities of mitochondria harboring the tRNA (Leu) (UUR) mutation in mitochondrial DNA derived from patients with maternally inherited diabetes and deafness (MIDD) and progressive kidney disease. *Diabetologia* 42:485–492, 1999
41. Calabrese V, Scapagnini G, Giuffrida Stella AM, Bates TE, Clark JB: Mitochondrial involvement in brain function and dysfunction: relevance to aging, neurodegenerative disorders and longevity. *Neurochem Res* 26:739–764, 2001
42. Orth M, Schapira HA: Mitochondria and degenerative disorders. *Am J Med Genet* 106:27–36, 2001
43. Hollenbeck CB, Reaven GM: Treatment of patients with non-insulin-dependent diabetes mellitus: diabetic control and insulin secretion and action after different treatment modalities. *Diabet Med* 4:311–316, 1987
44. Zoratti M, Szabo I: The mitochondrial permeability transition. *Biochim Biophys Acta* 1241:139–176, 1995
45. Moreira PI, Sancha MS, Moreno A, Oliveira C: Amyloid β-peptide promotes permeability transition pore in brain mitochondria. *Biosci Rep* 21:789–800, 2001
46. Moreira PI, Sancha MS, Rego AC, Moreno A, Oliveira C: Effect of amyloid β-peptide on the permeability transition pore: a comparative study. *J Neurosci Res* 69:257–267, 2002
47. Murphy AN, Bredesen DE, Cortopassi G, Wang E, Fiskum G: Bcl-2 potentiates the maximal calcium uptake capacity of neuronal cell mitochondria. *Proc Natl Acad Sci U S A* 93:9893–9898, 1996
48. Dubinsky JM, Levi Y: Calcium-induced activation of the mitochondrial permeability transition in hippocampal neurons. *J Neurosci Res* 53:728–741, 1998
49. Brustovetsky N, Dubinsky J: Dual responses of CNS mitochondria to elevated calcium. *J Neurosci* 20:103–113, 2000
50. Craft S, Newcomer J, Kanne S, Dagogo-Jack SE, Cryer P, Sheline Y, Luby J, Dagoogo-Jack A, Anderson A: Memory improvement following induced hyperinsulinaemia in Alzheimer's disease. *Neurobiol Aging* 17:123–130, 1996
51. Piert M, Koeppe RA, Giordani B, Berent S, Kuhl DE: Diminished glucose transport and phosphorylation in Alzheimer's disease determined by dynamic FDG-PET. *J Nucl Med* 37:201–208, 1996
52. Smyth MD, Kesslak JP, Cummings BJ, Cotman CW: Analysis of brain injury following intrahippocampal administration of beta-amyloid in streptozotocin-treated rats. *Neurobiol Aging* 15:153–159, 1994
53. Messier C, Gagnon M: Glucose regulation and cognitive functions: relation to Alzheimer's disease. *Neurobiol Aging* 18:153–159, 1996