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

Paula I. Moreira,1 Maria S. Santos,1 António M. Moreno,1 Raquel Seiça,2 and Catarina R. Oliveira2

This study evaluated the respiratory indexes (respiratory control ratio [RCR] and ADP/O ratio), mitochondrial transmembrane potential (∆Ψm), 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 Aβ25–35 or 2 μmol/l Aβ1–40, 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 Ca2+ (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 Aβ25–35 or 2 μmol/l Aβ1–40 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). Partridge et al (3) showed the existence of insulin receptors in the endothelium of the human brain-blood 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 (AB) on the function of brain mitochondria isolated from diabetic GK
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.** Asp₂⁻⁻ and Asp¹⁻⁻ were obtained from Bachem AG (Bubendorf, Germany). Protease (Sautterlin Carbaryl) 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 (HbA₁c, 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 HbA₁c levels were determined through ionic change monitored by a glucose oxidase reaction, using a glucometer and compatible chemicals. The supernatants were neutralized with 10 mol/l KOH in 5 ml of 0.05 mol/l perchloric acid. The supernatants were neutralized with 10 mol/l KOH in 5 ml of 0.05 mol/l Tris and centrifuged at 14,000 rpm for 5 min. The reaction 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 isotropic elution with 100 mmol/l phosphate buffer (KH₂PO₄; 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 HbA₁c levels in Wistar control and GK rats.** For confirming diabetes in GK rats, glycemia and the glycated hemoglobin (HbA₁c, GHb) levels were determined (Table 1). The percentage of hemoglobin in glycated form (GHb and HbA₁c) 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 significantly 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 after ADP has been consumed) and 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 Aβ₂₅₋₃₅ or 2 μmol/l Aβ₄₀ for 5 min before succinate addition, the ADP/O ratio also significantly increased: the decreases on RCR (Fig. 1A) and ADP/O ratio (Fig. 2A) were potentiated. A similar pattern occurred in Wistar control rats (Fig. 1B and 2B).

**TABLE 1**

<table>
<thead>
<tr>
<th></th>
<th>Wistar control (1.5 months; n = 8)</th>
<th>GK (1.5 months; n = 8)</th>
<th>Wistar control (12 months; n = 7)</th>
<th>GK (12 months; n = 7)</th>
<th>Wistar control (24 months; n = 5)</th>
<th>GK (24 months; n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycemia (mg/ml)</td>
<td>111.4 ± 9.9</td>
<td>223.6 ± 27.1</td>
<td>102.0 ± 4.2</td>
<td>256.8 ± 13.5</td>
<td>85.5 ± 6.5</td>
<td>180.5 ± 44.1</td>
</tr>
<tr>
<td>HbA₁c (%)</td>
<td>6.3 ± 0.7</td>
<td>6.0 ± 0.3</td>
<td>5.2 ± 0.1</td>
<td>9.6 ± 0.5</td>
<td>5.2 ± 0.4</td>
<td>7.7 ± 0.3</td>
</tr>
<tr>
<td>GHb (%)</td>
<td>7.2 ± 0.9</td>
<td>6.6 ± 0.5</td>
<td>6.1 ± 0.1</td>
<td>12.6 ± 0.8</td>
<td>5.9 ± 0.6</td>
<td>9.8 ± 0.4</td>
</tr>
</tbody>
</table>

Data are the means ± SE. HbA₁c, and GHb data are expressed as a percentage of the total hemoglobin. *P < 0.001; **P < 0.01; ***P < 0.05, when compared with mitochondria isolated from 1.5-month-old Wistar control rats. *P < 0.001, **P < 0.01; ***P < 0.05, when compared with mitochondria isolated from 1.5-month-old GK rats. *P < 0.01, when compared with mitochondria isolated from 12-month-old Wistar control rats. *P < 0.01; **P < 0.05; 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 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 (ΔpH) and a voltage gradient (ΔΨm) across the mitochondrial inner membrane. Figure 3 is a representative trace of the effect of Aβ 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 Aβ 25-35 or 2 μmol/l Aβ 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 Aβ 25-35 or 2 μmol/l Aβ 1-40 led to a significant decrease of both parameters. In the presence of 2 μmol/l Aβ 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.
As shown in Fig. 4A, an age-related decrease in ATP/ADP ratio of diabetic brain mitochondria was observed. The presence of 50 μmol/l Aβ_{25-35} or 2 μmol/l Aβ_{1-40} exacerbated the decrease on ATP levels, this effect being more pronounced in the presence of 2 μmol/l Aβ_{1-40}. A similar pattern occurred with Wistar control rats (Fig. 4B). 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 Aβ_{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. 5A).

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. 5B and 5C). 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. 5B). In mitochondria isolated from 24-month-old rats, Aβ_{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 Aβ_{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. 7A). 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. 7B).

**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 (HbA1C 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
Dysfunction of the mitochondrial respiratory chain has been described in diabetes (38). Accordingly, our data show an age-related impairment of mitochondrial function in GK rats, which is associated with a decrease in OXPHOS efficiency and action (38). The specific mitochondrial enzymes (39) involved in the maintenance of cellular energy metabolism are known to be altered in diabetes, resulting in a decrease of the activity of both enzymes. The effect of age and Aβ on mitochondrial ATP/ADP ratio is shown in Table 2.

![Diagram](image)

**Table 2**

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>ATP/ADP (% of control)</th>
<th>ATP/ADP (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.5</td>
<td>12</td>
</tr>
<tr>
<td>1.5-month-old</td>
<td>24</td>
<td>24</td>
</tr>
</tbody>
</table>

**FIG. 4** Effect of age and Aβ on mitochondrial ATP/ADP ratio, phase, and repolarization level.
the respiratory chain (Fig. 1A) and the uncoupling of OXPHOS (Fig. 2A) are not accompanied by a decrease in ∆Ψm (Table 2). However, an age-related decrease in ATP/ADP ratio of mitochondria isolated from diabetic rats occurred (Fig. 4A). 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. 1A, 2A, and 4A) when compared with Wistar control rats of the same age (Figs. 1B, 2B, and 4B). 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 Ca2+ addition, 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 Ca2+ 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 Ca2+ 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. 5. Effect of age on PTP induction: susceptibility to Ca2+ 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. Ca2+ was added 1.5 min after mitochondria energization. The traces are typical of three experiments.](image-url)
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, \( \beta \)-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 \( \beta \)-amyloid, 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.

ACKNOWLEDGMENTS

This work was supported by FCT (Portuguese Research Council). P.M. is the recipient of grant SFRH/BD/5320/2001.

REFERENCES


AGING AND Αβ INDUCE MITOCHONDRIA IMPAIRMENT


34. 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


