Enhanced permeability transition explains the reduced calcium uptake in cardiac mitochondria from streptozotocin-induced diabetic rats

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Received 4 August 2003; revised 4 September 2003; accepted 21 October 2003

First published online 30 October 2003

Edited by Vladimir Skulachev

Abstract Cardiac dysfunction is associated with diabetes. It was previously shown that heart mitochondria from diabetic rats have a reduced calcium accumulation capacity. The objective of this work was to determine whether the reduction in calcium accumulation by cardiac mitochondria from diabetic rats is related to an enhanced susceptibility to induction of the mitochondrial permeability transition. Streptozotocin-induced diabetic rats were used as a model to study the alterations caused by diabetes in the permeability transition, 21 days after streptozotocin administration. Heart mitochondria were isolated to evaluate respiratory parameters and susceptibility to the calcium-dependent permeability transition. Our results show that streptozotocin diabetes facilitates the mitochondrial permeability transition in cardiac mitochondria, resulting in decreased mitochondrial calcium accumulation. We also observed that heart mitochondria from diabetic rats had depressed oxygen consumption during the phosphorylative state. The reduced mitochondrial calcium uptake observed in heart mitochondria from diabetic rats is related to an enhanced susceptibility to the permeability transition rather than to damage to the calcium uptake machinery.

Key words: Mitochondrion; Diabetes; Heart; Streptozotocin; Permeability transition

1. Introduction

Diabetes mellitus is one of the most common metabolic diseases in the world. The complications associated with this disease are often responsible for a decreased quality of life in many patients. The diabetic population has a greater probability to suffer from cardiovascular problems and heart failure than the general population.

The role of mitochondria in the development of cardiac dysfunction has not been studied in detail, despite some experiments conducted in isolated liver mitochondria [1]. In heart mitochondria, streptozotocin (STZ)-induced diabetes was shown to impair mitochondrial oxidative phosphorylation and calcium loading capacity, in close relation to observed diastolic dysfunction [2]. The results were confirmed by Tanaka et al. [3] using permeabilized myocytes from STZ-injected animals. One of the negative conditions that can affect mitochondrial function is the mitochondrial permeability transition (MPT), caused by the formation of polyprotein pores (MPTP), occurring with mitochondrial calcium overload and oxidative stress, and leading to mitochondrial dysfunction and even cell death [4].

The aim of this work was to determine differences in the susceptibility to the MPT in heart mitochondria from STZ-induced diabetic and control animals, something that was previously never demonstrated. Our starting hypothesis was that the reduced mitochondrial calcium uptake observed in STZ-induced diabetic rat hearts [2,3] was due to enhanced MPT induction, with all the consequences to cardiac cell associated with that condition.

2. Materials and methods

2.1. Materials

All reagents and chemicals used were of the highest grade of purity commercially available. Calcium Green 5-N was obtained from Molecular Probes (Eugene, OR, USA).

2.2. Animals

Male Wistar rats (2–3 months) were maintained in our local colony (Laboratory Animal Research Center, University Hospitals, Coimbra) with food (URF1, Charles River, France) and water at pH 5.5 (except during fasting periods). In this study, the 'Principles of Laboratory Animal Care' (NIH publication no. 85-23, revised 1996) were followed.

2.3. Induction and characterization of STZ-induced diabetes

Diabetes was induced using STZ, as previously described [5]. Rats (2–3 months) were randomly divided into two groups of five animals each. Diabetes was induced in one group of animals after a 16 h fasting period with a single i.p. STZ injection, 50 mg/kg. STZ was dissolved in citrate 100 mM, pH 4.5. Control animals were injected with citrate. In the following 24 h, animals were orally fed with glycosylated serum in order to avoid hypoglycemia resulting from massive destruction of β-cells and release of intracellular insulin associated with STZ treatment [5]. Animals were kept for 21 days before the experiments. Blood glucose concentration was determined from the tail vein using a commercial glucometer (Glucometer-Elite, Bayer). Glycated hemoglobin (HbA_{1c}) was determined at the time of sacrifice through ionic exchange chromatographic assay (Abbott IMx Glicohemoglobin, Abbott Laboratories, Portugal).

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Abbreviations: $\Delta\Psi$, mitochondrial electrical transmembrane potential; STZ, streptozotocin; RCR, respiratory control ratio; MPT(P), mitochondrial permeability transition (pore); TPP^+, tetraphenylphosphonium cation

0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)01233-X

2.4. Isolation of mitochondria from rat heart

Rat heart mitochondria were prepared as previously described [6].

2.5. Measurement of mitochondrial transmembrane potential

The mitochondrial transmembrane potential ($\Delta\Psi$) was estimated with a tetraphenylphosphonium cation (TPP^+) electrode as described in [6], at 25°C in 2 ml of the reaction medium (200 mM sucrose, 10 mM Tris–MOPS, 10 μM EGTA, 5 mM KH_2PO_4 and 2 μM rotenone, pH 7.4) supplemented with 1 μM TPP^+, 0.25 μg oligomycin and 0.5 mg of mitochondria. Mitochondria were energized with 4 mM succinate.

2.6. Mitochondrial oxygen consumption

Oxygen consumption of isolated heart mitochondria was monitored polarographically with a Clark oxygen electrode. Reactions were carried out, at 25°C, in 2 ml of the reaction medium (without TPP^+ and with 4 mM succinate). Mitochondria were suspended at a concentration of 0.5 mg/ml in the respiratory medium. ADP (100 nmol) was added to induce state 3 respiration. The respiratory control ratio (RCR) was calculated as the ratio between state 3 and state 4 respiration. The ADP/O was calculated as the number of nmol ADP phosphorylated per natom oxygen consumed.

2.7. Extramitochondrial calcium movements

Extramitochondrial free Ca^{2+} was measured with the fluorescence probe Calcium Green 5-N. Heart mitochondria (0.2 mg) were suspended in 2 ml of buffer containing 200 mM sucrose, 10 mM Tris, 10 μM EGTA, 1 mM KH_2PO_4, 2 μM rotenone, 4 mM succinate, 0.25 μg oligomycin/mg protein and 100 nM Calcium Green 5-N. Fluorescence was recorded continuously at 25°C using a Perkin-Elmer LS-50B fluorescence spectrometer with excitation and emission wavelengths of 506 and 531 nm, respectively. Due to the lower amount of protein used, the amount of phosphate in the reaction buffer was reduced to 1 mM in comparison with the 5 mM used for $\Delta\Psi$ measurements.

2.8. Statistical analysis

The results are presented as mean ± S.E.M. of five animals for each group. Comparisons between the groups were made using one-way analysis of variance two-tailed unpaired *t*-tests. A P value < 0.05 was considered statistically significant.

3. Results

We observed that the control group had a time-dependent relative weight gain, in contrast to the STZ group, which lost body weight (137.0 ± 17.8 vs. 92.8 ± 3.0%, $P < 0.05$, $n = 5$ each), probably due to loss of body water. There was also a sharp increase in the levels of blood glucose in the STZ group, reaching a plateau approximately 3 days after administration, with an average value of 469 mg/dl (vs. 90.4 ± 6.3, $P < 0.0001$, $n = 5$ each). The levels of HbA_{1c} showed that the STZ group possessed increased levels of these glycated hemoglobin derivatives (11.28 ± 0.27 vs. 5.73 ± 0.12, $P < 0.0001$, $n = 5$ each).

As the experiments involving MPTP susceptibility were done with succinate-energized mitochondria, our bioenergetic parameters were only tested with that substrate. No differences were found when measuring state 4 respiration (67.3 ± 0.7 for the control group vs. 55.3 ± 6.4 for the STZ group, values in natoms O/min/mg protein, $n = 5$ each). A significant difference in state 3 respiration (ADP-stimulated respiration) was found in between the two groups (218.9 ± 8.3 for the control group vs. 160.6 ± .17.2 for the STZ group, values in natoms O/min/mg protein, $P < 0.05$, $n = 5$ each). No significant differences were found in all other parameters, including the ADP/O ratio (1.62 ± 0.06 for the control group vs. 1.68 ± .0.19 for the STZ group, $n = 5$ each) and the RCR (2.85 ± 0.25 vs. 3.25 ± 0.12, $n = 5$ each).

After a calcium pulse, heart mitochondria from the control group were better able to sustain the initial $\Delta\Psi$ value than mitochondria from the diabetic STZ group (Fig. 1, upper panel). Heart mitochondria from the STZ group were not able to repolarize and suffered a complete depolarization prevented by cyclosporin A (Fig. 1, lower panel). As seen in Fig. 1, lower panel, prior to calcium addition, no differences in $\Delta\Psi$ were observable between both groups, even in the presence of cyclosporin A. A direct visualization of mitochondrial calcium uptake and release was performed using an extramitochondrial fluorescent calcium probe (Fig. 2). After a calcium pulse (700 nmol/mg protein), mitochondria from both groups accumulated the same amount of calcium. Following that, mitochondria from the STZ group were not able to retain the accumulated calcium, and began releasing it to the buffer. In the presence of cyclosporin A, both types of mitochondria were able to retain the same amount of calcium. Sixteen hundred seconds after the start of each experiment, extramitochondrial calcium levels were much higher in heart mitochondria from STZ-injected animals. Taken together, these results indicate that heart mitochondria from STZ-induced diabetic rats have a depressed capacity to accumulate calcium, by way of an enhanced sensitivity to induction of MPT.

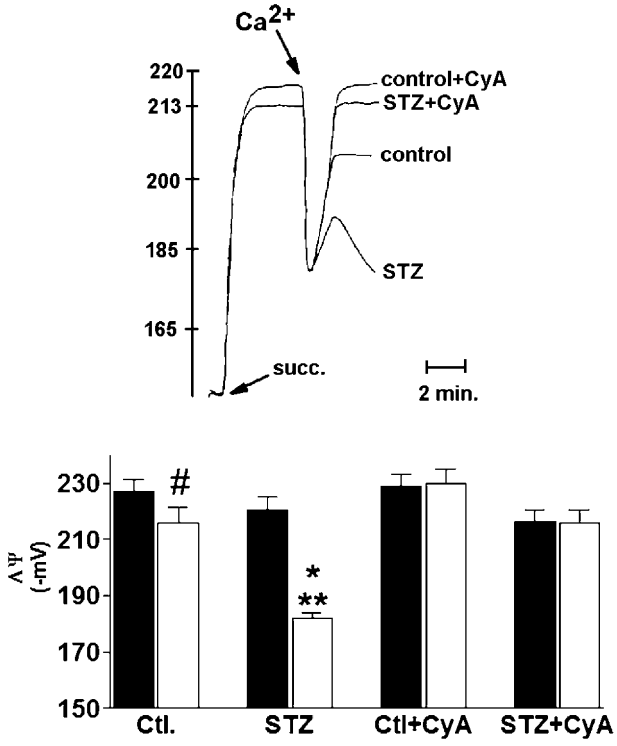

Fig. 1. Upper panel: Representative recording of mitochondrial electric potential measured by a TPP^+-selective electrode ($n = 5$ each). Calcium (800 nmol/mg protein) was added in order to induce the MPTP. $\Delta\Psi$ was calculated without correction for TPP^+ passive binding to mitochondrial membranes. 0.5 mg mitochondrial protein was resuspended in 2 ml reaction medium (200 mM sucrose, 10 mM Tris, 10 μM EGTA and 5 mM KH_2PO_4, supplemented with 2 μM rotenone, 0.25 μg oligomycin and 1 μM TPP^+). Mitochondrial energization was achieved with 4 mM succinate. Cyclosporin A (CyA, 1 μM) was added to the mitochondrial suspension before energization. Numbers on the ordinates represent mitochondrial $\Delta\Psi$ (−mV). Lower panel: statistical treatment of data. Displayed are the $\Delta\Psi$ values prior to calcium addition (black bars) and the highest $\Delta\Psi$ value reached by mitochondrial after calcium addition (white bars). $^*P < 0.001$ vs. pre-calcium STZ, $^{**}P < 0.001$ vs. STZ+CyA, $^{\#}P < 0.001$ vs. post-calcium STZ, $n = 5$ each.

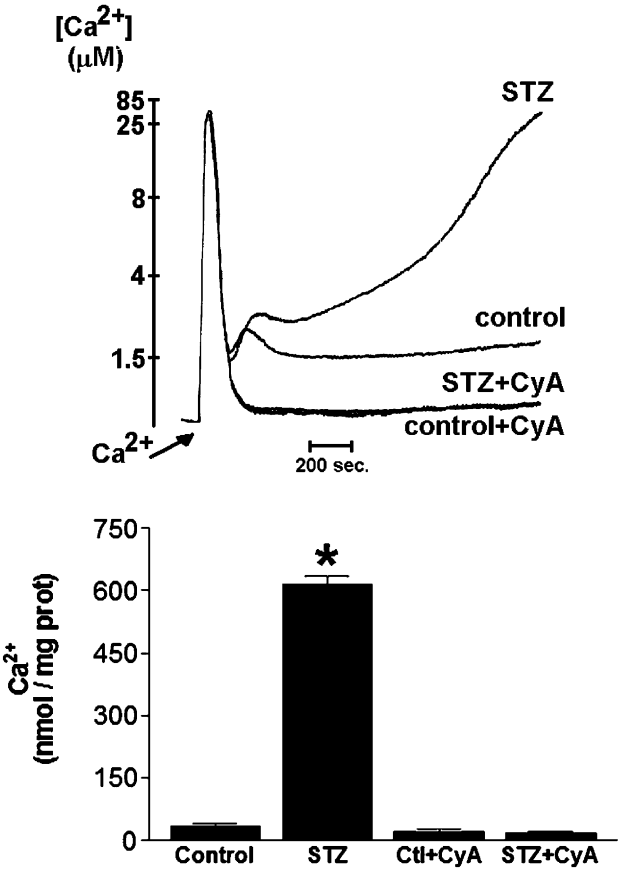

Fig. 2. Upper panel: Representative recording of extramitochondrial calcium movements measured using the fluorescent calcium-sensitive probe Calcium Green 5-N ($n = 5$ each). 0.2 mg of protein was resuspended in 2 ml of medium containing 200 mM sucrose, 10 mM Tris, 10 μM EGTA, 1 mM KH_2PO_4, 2 μM rotenone and 0.05 μg oligomycin. Mitochondrial energization was achieved with 4 mM succinate. A pulse of 700 nmol calcium per mg protein was added to the mitochondrial suspension. Free extramitochondrial Ca^{2+} was monitored with 100 nM Calcium Green 5-N. EGTA was added at the end of each experiment to calculate the basal line. Extramitochondrial calcium was determined as described in Section 2. Cyclosporin A (CyA, 0.2 μM) was added before calcium addition. Lower panel: Extramitochondrial calcium concentration measured 1600 s after the start of each experiment. $^*P < 0.001$ vs. control and vs. STZ+CyA, $n = 5$ each.

4. Discussion

A relationship between dysfunctional cardiac work and defective mitochondrial function associated with diabetes has been previously reported [2]. This elegant study showed a reduced calcium uptake in heart mitochondria from STZ-injected rats, although the primary reason was not found. STZ is an established animal model to generate diabetic cardiomyopathy [5]. In another model of STZ-induced diabetic mitochondriopathy, Tanaka et al. [3] also showed a reduced mitochondrial calcium accumulation in permeabilized myocytes, although the reason was again not described.

The hypothesis for this work was that the MPT, a deleterious condition triggered by excessive calcium and oxidative stress [4], could account for the decreased calcium uptake by heart mitochondria from diabetic rats [2,3].

Our results revealed that both groups of mitochondria accumulated the same amount of calcium in the presence of cyclosporin A. This compound, a known MPTP inhibitor [7], inhibited the $\Delta\Psi$ depolarization after calcium uptake and allowed mitochondria from STZ rats to accumulate calcium, identically to control mitochondria. The data suggest that the depressed calcium uptake was not due to dysfunctional calcium uptake machinery, nor to enhanced calcium release by MPT-independent processes, which is in agreement with previous reports [2]. Heart mitochondria from the STZ group also showed a lower state 3 respiration. The unchanged ADP/O value suggests that the mitochondrial phosphorylative machinery may not have been affected in our model. Instead, the result suggests alterations at the level of the respiratory chain. Kristal et al. [1] observed a defect in hepatic mitochondrial complex III of STZ-injected rats. Kucharska et al. [8] demonstrated a deficit of coenzyme Q in heart mitochondria of rats with STZ-induced diabetes. The described alterations, although not apparent during mitochondrial state 4, may hinder the maximal rate of state 3 respiration in our STZ experimental model.

The enhanced MPT induction and reduced state 3 respiration hint that heart mitochondria from STZ-injected animals may suffer from two distinct functional defects, depending on the presence of calcium. The lower state 3 occurred under conditions in which extramitochondrial calcium levels were very low or absent (as the experiments were made in a buffer containing EGTA and in the absence of externally added calcium). When calcium was added, a new defective mitochondrial function was revealed in diabetic animals: a lower calcium loading capacity. Under the conditions we used for measuring mitochondrial oxygen consumption, MPT induction is supposedly not a factor due to the absence of calcium. Nevertheless, it cannot bee excluded that in vivo, other alterations in mitochondrial oxygen consumption parameters may occur (like a decreased ADP/O ratio or increased state 4 respiration), especially when heart mitochondria are called to deal with above-physiological calcium concentrations with consequent increased MPT induction. Nevertheless, the reduced respiration during the phosphorylation state does not require the presence of calcium and may be present under resting (physiological) conditions.

The results of this study also appear to be contradictory with previous reports from our group when using another animal model for diabetes, the Goto-Kakizaki (GK) rat [9]. We showed that heart mitochondria from GK rats possess a decreased susceptibility to the MPT. Nevertheless, there are differences between both types of animals and the transposition to a clinical context may not be immediate. The average blood glucose levels are much smaller in the GK rat than in the STZ rat, so GK rats are considered to display a milder and hereditary form of diabetes ([9] and references therein). In fact, the GK rat much better resembles the typical diabetic patient with absent or mild chronic cardiovascular problems, while the STZ animal model (widely studied in the literature) should be regarded as a model for short-term hyperglycemic crisis or for late forms of severe diabetes (and not for cardiac dysfunction of the typical diabetic patient). In this context, the relation between differences in the susceptibility to the MPT and the severity of the hyperglycemic episode may help explain contradictory results in the literature, concerning the resistance of the diabetic heart to acute ischemia–reperfusion damage [10,11]. In fact, the authors stated that the resistance

to cardiac ischemia and reperfusion appears to be directly related to the severity of the diabetic condition.

The link between different hyperglycemia levels and differential MPTP induction has still to be explored. Diabetes is often associated with increased oxidative stress [12], a condition known to induce the MPT via the oxidation of critical thiol residues (for a review, see [4]). In fact, oxidative stress was already observed in the hearts of STZ-injected animals [13,14]. We can speculate that the defect found in mitochondrial complex III by Kristal et al. [1] may, in fact (as the authors proposed) lead to increased reactive oxygen species generation by the mitochondrial respiratory chain, a fact that could be exacerbated by the presence of calcium. The absence of differences in mitochondrial $\Delta\Psi$ before calcium addition (Fig. 1, pre-calcium) led us to think that a possible voltage difference between the experimental groups was not a cause for differential MPT gating.

Sustained enhancement of MPTP openings in heart mitochondria from STZ-injected animals may induce a decrease in mitochondrial ATP production, large amplitude swelling as well as cell death and reduced resistance to acute ischemia followed by reperfusion [4]. It is also known that constant oxidative stress may lead to the activation of one potential mechanism for mitochondrial defense, the so-called mitochondrial mild uncoupling, in which small decreases of membrane potential, not enough to hinder ATP production, may decrease the amount of free radicals produced by the respiratory chain [15]. We may speculate that a low conductance form of the MPT may exist in vivo in STZ-injected animals, leading to mild mitochondrial uncoupling as an ultimate defense. Constant oxidative stress and MPT activation would later lead to irreversible mitochondrial and cellular damage, with the consequences described above. In our experimental conditions, and in the absence of calcium, no significant decrease in mitochondrial $\Delta\Psi$ in succinate-energized mitochondria was observed, suggesting that the mild uncoupling mechanism may not be fully functional in the absence of calcium.

Our results are also in contradiction with the ones obtained by Kristal et al. [16]. The authors observed that the MPT was decreased in the livers of STZ-induced diabetic rats. There is no unique explanation for this. Heart and liver possess different signaling pathways and antioxidant defense reserves and so may respond differently to hyperglycemia and oxidative stress. This may explain why the heart is primarily affected during the course of diabetes.

The results may also contribute to explain why severe hyperglycemia was observed to lead to mitochondria-mediated cell death [17]. In opposition, mild chronic glycemia (as in GK rats) appears to up-regulate anti-apoptotic proteins [18], which may represent an adaptation response to a chronic (but again, mild) condition. A possible relation between the increased expression of anti-apoptotic proteins and MPTP induction has not been explored yet in the context of milder forms of diabetes.

Our observations offer important insights, not only to explain the reduced resistance to cardiac ischemia and reperfusion observed in patients with more severe forms of diabetes, but also for the design of new therapeutic approaches to reduce mitochondrial dysfunction and cell death typically associated with diabetes.

Acknowledgements: We are grateful to Prof. João Patrício and coworkers (Maria de Lourdes and Carlos Silva, Animal Research Center Laboratory, University Hospitals, Coimbra) for all work concerning animal care. P.J.O. and A.P.R. are supported by Ph.D. grants from the Portuguese Foundation for Science and Technology (PRAXIS XXI/BD/21494/99 and PRAXIS XXI/BD/21454/99, respectively).

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