ORIGINAL ARTICLE

Mitochondrial Complex I, H_2O_2 and NO as Prodromal Signals of Cardiac Dysfunction in Type 1 Diabetes

Complejo I, H_2O_2 y NO mitocondriales como señales prodrómicas de la disfunción cardíaca en diabetes tipo 1

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ABSTRACT

Background: Previous results from our laboratory suggest that heart mitochondrial dysfunction precedes myocardial failure associated with sustained hyperglycemia.
Purpose: The aim of this study was to analyze the early events that take place in heart mitochondria in a type 1 diabetes mellitus (DM) model.
Methods: Male Wistar rats were injected with streptozotocin (STZ; 60 mg/kg, ip.) to induce DM. They were euthanized 10 or 14 days later and the heart mitochondrial fraction was obtained.
Results: State 3 O_2 consumption in the presence of malate-glutamate (21%) or succinate (16%), and complex I-III (27%), II-III (24%) and IV (22%) activities were lower in diabetic animals 14 days after STZ injection. When animals were euthanized at day 10, only state 3 O_2 consumption sustained by complex I substrates (23%) and its corresponding respiratory control (30%) were lower in rats injected with STZ, in agreement with reduced complex I-III activity (17%). These changes were accompanied by increased H_2O_2 (117%), NO (30%) and $ONOO^-$ (~225%) production rates, mtNOS expression (29%) and O_2^- (~150%) and NO (~30%) steady-state concentrations, together with a decrease in Mn-SOD activity (15%) and mitochondrial [GSSG+GSH] (28%), without changes in PGC-1α expression.
Conclusion: Complex I dysfunction and increased H_2O_2, NO and $ONOO^-$ production rates can be considered subcellular prodromal signals of the mitochondrial damage that precedes myocardial dysfunction in diabetes.

Key Words: Type 1 Diabetes Mellitus - Hyperglycemia - Mitochondrial Complex I - Heart mitochondria - Nitric Oxide - Hydrogen Peroxide

RESUMEN

Introducción: Resultados del laboratorio sugieren que la disfunción mitocondrial en el corazón precede a la falla miocárdica asociada a la hiperglucemia sostenida.
Objetivo: Estudiar los eventos tempranos que ocurren en las mitocondrias de corazón en un modelo de diabetes mellitus (DM) tipo 1.
Materiales y métodos: Ratas Wistar macho fueron inyectadas con estreptozotocina (STZ; 60 mg/kg, ip) y sacrificadas 10 o 14 días posinyección. Se obtuvo la fracción mitocondrial de corazón.
Resultados: El consumo de O_2 en estado 3 en presencia de malato-glutamato (21%) o succinato (16%) y las actividades de los complejos I-III (27%), II-III (24%) y IV (22%) fueron menores en los animales diabéticos a los 14 días posinyección. Cuando los animales se sacrificaron al día 10, solo el consumo de O_2 en estado 3 en presencia de sustratos del complejo I (23%) y su control respiratorio (30%) fueron menores en las ratas inyectadas con STZ, de acuerdo con una reducción en la actividad del complejo I-III (17%). Estos cambios se acompañaron de un aumento en las velocidades de producción de H_2O_2 (117%), NO (30%) y $ONOO^-$ (~225%), en la expresión de mtNOS (29%) y en la $[O_2^-]$ss (~150%) y [NO]ss (~30%), junto con una disminución de la actividad de la Mn-SOD (15%) y la [GSSG+GSH]mitocondrial (28%), sin cambios en la expresión de PGC-1α.
Conclusión: La disfunción del complejo I y el aumento en la generación de H_2O_2, NO y $ONOO^-$ pueden considerarse señales subcelulares prodrómicas del deterioro de la función mitocondrial que precede a la disfunción cardíaca en la diabetes.

Palabras clave: Diabetes mellitus tipo 1 - Hiperglucemia - Complejo I mitocondrial - Mitocondrias cardíacas - Óxido nítrico - Peróxido de hidrógeno

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INTRODUCTION

Diabetes Mellitus is a chronic metabolic disease characterized by an increase in blood glucose levels, which could be developed as a result of defects in insulin secretion, insulin action, or both. Cardiovascular complications are the main cause of morbidity and mortality among the diabetic population (1, 2), with sustained hyperglycemia being one of the risk factor that ultimately leads to diabetic cardiomyopathy (3, 4). Although the latter is frequently associated with type 2 diabetes, patients with type 1 diabetes also suffer a metabolically induced cardiomyopathy, which occurs independently of hypertension, nephropathy, or ischemic heart disease (5, 6). Previous results from our laboratory, obtained using an experimental model of type 1 diabetes in rat, show that the sustained hyperglycemia for 25 days leads to generalized cardiac mitochondrial dysfunction, in the absence of hypertrophy and changes in resting cardiac function, but with cardiac compromise against a work overload (7, 8), suggesting that mitochondrial function impairment precedes myocardial failure in diabetic patients.

Considering that most of the published studies refer to cardiac mitochondrial function in models of type 2 diabetes–in association with insulin resistance, obesity, hypercholesterolemia and hypertension– or in type 1 diabetic models during very prolonged hyperglycemia, the purpose of this work was to study the early events that occur in rat heart mitochondria in an experimental model of type 1 diabetes.

METHODS

Male Wistar rats (200-250 g) were randomly divided into two groups: control (C) and diabetes mellitus (DM). Diabetes was induced by an intraperitoneal injection of streptozotocin (STZ, 60 mg/kg) diluted in citrate buffer 100 mM pH 4.50. Group C animals received an intraperitoneal injection of the vehicle (citrate buffer 100 mM pH 4.50). Glycemia was determined 3 days after STZ injection and throughout the experimental protocol, using a One Touch Ultra, Johnson & Johnson glucometer. Rats with blood glucose concentration >200 mg/dl 3 days after STZ injection were considered diabetic (9). Animal weight was assessed throughout the study period. At 10 or 14 days post-STZ injection, the animals were euthanized in a CO_2 atmosphere, the heart was removed, and the mitochondrial fraction was obtained by differential centrifugation (10).

Mitochondrial O_2 consumption was assessed in the presence of complex I (malate plus glutamate) or complex II (succinate) substrates, both in the absence (state 4 or passive respiration) as in the presence (state 3 or active respiration) of ADP using a Clark electrode (Hansatech Oxygraph, Hansatech Instruments Ltd, Norfolk, England) (11). Respiratory control (RC) was calculated as state 3/state 4 O_2 consumption ratio. The respiratory complex I-III (NADH-cytochrome c reductase), II-III (succinate-cytochrome c reductase) and IV (cytochrome oxidase) activities were assessed at 550-540 nm with a diode array spectrophotometer (12).

The mitochondrial hydrogen peroxide (H_2O_2) production rate was measured fluorometrically at 365-450 nm using the scopoletin-horseradish peroxidase (HRP) method, in the presence of complex I substrates (malate plus glutamate) (12, 13). Mitochondrial nitric oxide (NO) production was determined through the oxidation of oxyhemoglobin to methemoglobin with a diode array spectrophotometer, at 577-591 nm (12, 14, 15). Mn-superoxide dismutase (Mn-SOD) activity was assessed spectrophotometrically from the ferricytochrome c to ferrocytochrome c reduction rate by the superoxide anion ($O_2^{\cdot-}$) using the xanthine-xanthine oxidase system (16). Steady state $O_2^{\cdot-}$ and NO concentrations ($[O_2^{\cdot-}]ss$ and [NO]ss) were calculated in the mitochondrial matrix (8) from the experimental measurements of H_2O_2, NO and Mn-SOD, and the peroxynitrite ($ONOO^-$) production rate was estimated from these concentrations. Mitochondrial concentrations of reduced (GSH) and oxidized (GSSG) glutathione were determined by micro HPLC-MS/MS (17). Mitochondrial nitric oxide synthase (mtNOS) and peroxisomal proliferation-activated receptor γ coactivator 1α (PGC-1α) expressions were analyzed by western blot using neuronal anti-NOS (H299: sc-8309, Santa Cruz Biotechnology) and anti-PGC-1α (K-15: sc-5816, Santa Cruz Biotechnology) antibodies, respectively. Anti-VDAC-1 (D-16: sc-32063, Biotechnology) and anti-β-tubulin ([1E1-E8-H4] ab131205, Abcam) antibodies were used as loading controls.

Statistical analysis

The results included in tables and figures are expressed as mean±SEM and represent repetitions of 3 to 10 independent experiments. Statistical analysis was performed using the GraphPad Instat 4 program (GraphPad Software, La Jolla, CA, USA). Student's t test was used to analyze significant differences between C and DM groups, and ANOVA-Dunnett's test to analyze the significant differences of group C or group DM with respect to treatment onset (day 0).

Ethical considerations

The experimental protocol was approved by the Institutional Committee for the Care and Use of Laboratory Animals of the School of Pharmacy and Biochemistry of University of Buenos Aires (CICUAL; 00663658/15) and the procedures were carried out following the indications of the Guide for the Care and Use of Laboratory Animals published by the United States National Academy of Sciences.

RESULTS

Glycemia assessed on the third day after STZ injection was significantly higher in diabetic animals compared with the C group and remained elevated throughout the experimental period, with no differences within the DM group throughout the treatment period (Fig. 1A).

The weight of the heart of STZ-treated animals at 10 days and 14 days post-injection was 15% and 25% lower respectively compared with the weight of control animals, in agreement with the lower increase in body weight observed in diabetic rats with respect to the normal growth profile of the C group (Fig. 1B). Consequently, there were no differences in the cardiac index (heart weight/body weight ratio) between the groups (C: 0.340±0.010; DM-10 days: 0.336±0.004; DM-14 days: 0.339±0.003), indicating the absence of hypertrophy at these experimental times.

In order to evaluate the cardiac mitochondrial function, O_2 consumption in the presence of malate-glutamate or succinate, as well as respiratory complex-

es I-III, II-III and IV activities were measured (Table 1). No changes in state 4 (passive) mitochondrial respiration, sustained by malate-glutamate or succinate, were observed in the heart of diabetic rats compared with control animals, neither at 10 nor at 14 days after STZ injection. When the rats were euthanized on day 10, which corresponds to approximately 7 days of hyperglycemia, state 3 O_2 consumption (active respiration), sustained by malate-glutamate (23%), as well as its corresponding respiratory control (30%), were lower in the diabetic group, in agreement with the reduction in complex I-III (17%) activity, with complex I being the only modified respiratory complex at this stage. On the contrary, when the animals were euthanized 14 days after STZ injection, that is, after ~11 days of hyperglycemia, both state 3 O_2 consumption, sustained by malate-glutamate (21%) as state 3 respiration, sustained by succinate (16%), and respiratory complex I-III (27%), II-III (24%) and IV (22%) activities were lower in the diabetic group in relation to the values detected in the control group.

Since ~7 days of hyperglycemia led to an incipient mitochondrial dysfunction, evidenced both by the decrease in O_2 consumption in state 3 sustained by malate-glutamate as by the reduction of complex I-III activity, that experimental period was considered an early stage of cardiac mitochondrial dysfunction. For this reason, reactive oxygen and nitrogen species generation, the redox state and mitochondrial biogenesis were studied in the heart of animals euthanized 10 days after STZ injection.

The mitochondrial redox state was evaluated by measuring the concentration of mitochondrial glutathione and Mn-SOD activity, the main mitochondrial non-enzymatic and enzymatic antioxidants, respectively (Table 2). The mitochondrial concentration of total glutathione ([GSH+GSSG]) was 28% lower in diabetic animals compared with that determined in control rats, due both to a decrease in the concentration of reduced glutathione ([GSH]) as in the concentration of oxidized glutathione ([GSSG]), with no differences in the [GSH]/[GSSG] ratio. Mn-SOD activity was also

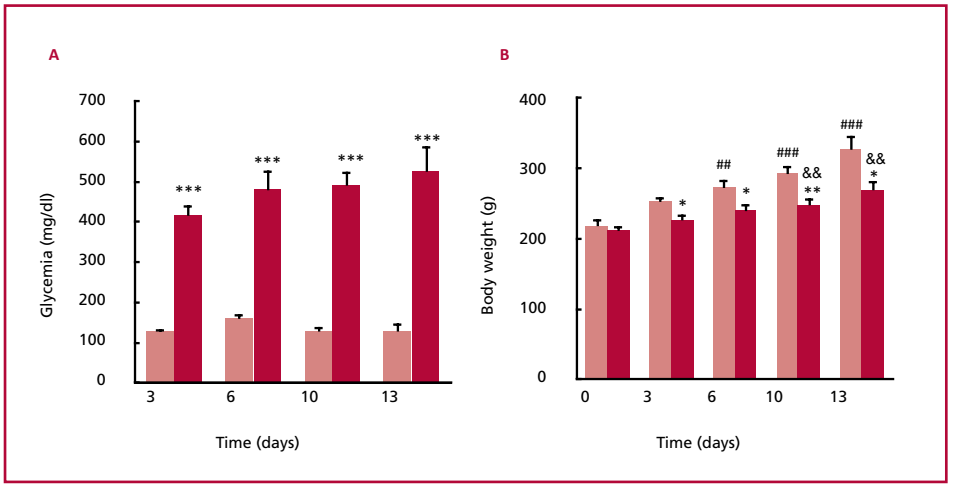

Fig. 1. Glycemia (A) and body weight (B) of the animals throughout the experimental protocol. Group C: pink bars; group DM: red bars.
*** p <0.005; ** p <0.01; * p <0.05 DM vs. C on the same treatment day (Student's t test); ### p <0.005; ## p <0.01 C vs. C at day 0 (ANOVA-Dunnett); && p <0.01 DM vs. DM at day 0 (ANOVA-Dunnett).

Table 1. Mitochondrial function. O_2 consumption and respiratory complexes in heart mitochondria of control and diabetic rats, euthanized 10 or 14 days after STZ injection. C: control, DM: diabetes mellitus

	C	DM	
		10 days	14 days
MITOCHONDRIAL O_2 CONSUMPTION			
MALATE-GLUTAMATE			
State 4 (ng-at. O/min.mg protein)	41 ± 3	45 ± 4	39 ± 3
State 3 (ng-at. O/min.mg protein)	353 ± 20	271 ± 24*	280 ± 18*
Respiratory control	8.6	6.0	7.2
SUCCINATE			
State 4 (ng-at. O/min.mg protein)	122 ± 5	126 ± 11	106 ± 13
State 3 (ng-at. O/min.mg protein)	347 ± 21	299 ± 23	290 ± 30
Respiratory control	2.8	2.4	2.7
RESPIRATORY COMPLEXES			
I-III (nmol/min.mg protein)	376 ± 14	312 ± 18*	274 ± 26***
II-III (nmol/min.mg protein)	184 ± 10	154 ± 16	140 ± 13*
IV (1/min.mg protein)	72 ± 4	69 ± 5	56 ± 4*

***p<0.005; *p<0.05 DM vs. C (Student's t test)

	C	DM - 10 days
[**Glutathione**] (pmol/mg protein)		
[GSH]	104 ± 5	76 ± 7
[GSSG]	8 ± 1	5.5 ± 0.7
[GSH+GSSG]	113 ± 6	81 ± 7*
[GSH]/[GSSG]	12 ± 1	14 ± 2
Mn-SOD		
Activity (USOD/mg protein)	79 ± 5	67.5 ± 2.5*
[Mn-SOD] (μM)	11.2 ± 0.8	9.8 ± 0.4

* p <0.05 DM vs. C (Student's t test)

Table 2. Mitochondrial redox state. [GSH], [GSSG] and Mn-SOD in hearts mitochondria of control and diabetic rats euthanized 10 days after STZ injection.
GSH: reduced glutathione
GSSG: oxidized glutathione
Mn-SOD: Manganese superoxide dismutase

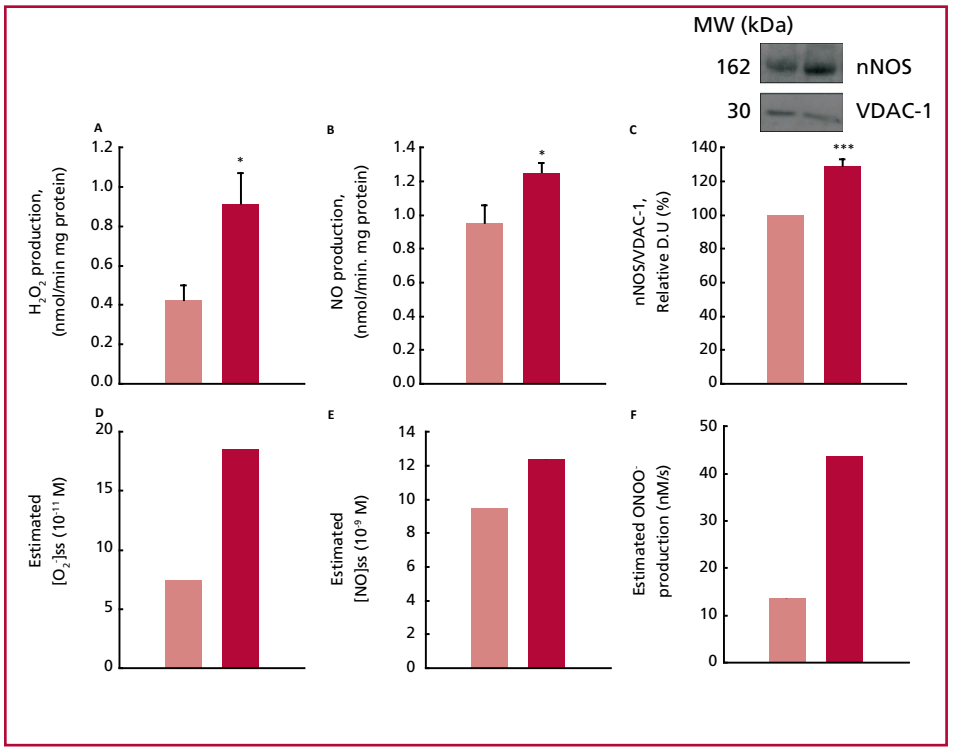

Fig. 2. Reactive oxygen and nitrogen species. Mitochondrial H_2O_2–malate+glutamate– (A), NO (B) and $ONOO^-$ production (F). mtNOS expression (C). Steady-state mitochondrial O_2^- (D) and NO concentrations (E). C group: pink bars; DM group: red bars.
*** p <0.005; * p <0.05 DM vs. C (Student's t test)
H_2O_2: hydrogen peroxide
NO: nitric oxide $ONOO^-$ peroxinitrite

found to be 15% lower in the heart mitochondria of diabetic animals euthanized 10 days after STZ injection.

Regarding the production rates of reactive oxygen species, the generation of H_2O_2 in the presence of complex I substrates was significantly higher (117%) in the heart mitochondria of diabetic animals after ~7 days of hyperglycemia, compared with that assessed in the control group (Fig. 2A). The rate of mitochondrial NO generation also increased by 30% (Fig. 2B), in agreement with 29% increase in the expression of NOS in mitochondrial membranes (mtNOS) (Fig. 2C).

The steady-state concentrations of O_2^- –stoichiometric precursor of H_2O_2– and NO were estimated from experimental measurements that have been performed in the heart mitochondria of diabetic animals and controls. A very marked increase (~150%) in $[O_2^-]$ss (Fig. 2D) was observed in the diabetic group compared with the control group, together with ~30% increase in [NO]ss (Fig. 2E). The rate of mitochondrial $ONOO^-$ generation (Fig. 2F) estimated from the steady state concentrations of these species, resulted 3 times higher in the heart of animals injected with STZ than in control rats.

Moreover, PGC-1α transcription factor expression, the main regulator of mitochondrial biogenesis, was determined in total heart homogenate from animals euthanized 10 days after STZ injection, and cardiac mitochondrial mass was estimated from the ratio of cytochrome oxidase (COX) activity assessed in the mitochondrial fraction and in the homogenate (18). No differences were found between the experimental groups, neither in PGC-1α expression (Fig. 3) nor in the cardiac mitochondrial mass (C: 24.6±0.1 and DM: 24.1±0.2 mg mitochondrial protein/g tissue), indicating that after 7 days of hyperglycemia, de novo mitochondria synthesis has not been triggered, contrary to what happens when hyperglycemia is sustained for 25 days (8).

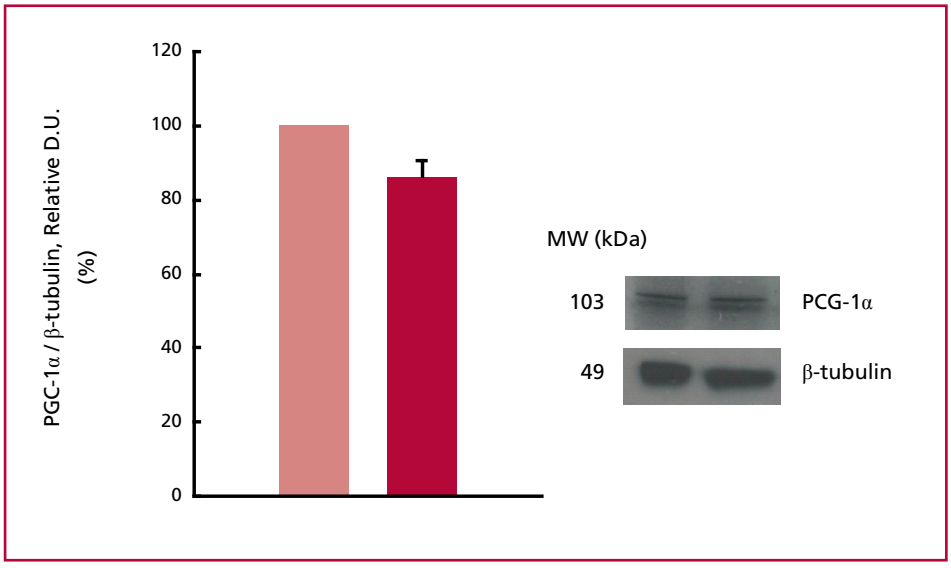

Fig. 3. Mitochondrial biogenesis. PGC-1α expression in heart homogenate from control and diabetic rats. Group C: pink bars; group DM: red bars.
PGC-1 α: peroxisomal proliferation-activated receptor gamma coactivator 1 α

DISCUSSION

The results show that ~7 days of sustained hyperglycemia are sufficient to show incipient cardiac mitochondrial dysfunction. The decrease in state 3 O_2 consumption and the enhancement in H_2O_2 production, in both cases when using malate-glutamate, in addition with the reduction of complex I activity, suggest the presence of structural modifications of complex I proteins in the first phase of cardiac dysfunction in diabetic rats. The increase in H_2O_2 production was accompanied by an increase in $ONOO^-$ generation, the latter at the expense of a marked increment in O_2^- concentration and in NO generation and mtNOS expression, the enzyme responsible for its synthesis. It should be mentioned that $ONOO^-$ is a highly oxidizing and nitrating species, involved in complex I inhibition through tyrosine nitration and/or nitrosylation. (12, 19). Since no changes were evidenced in PGC-1α factor expression or in cardiac mitochondrial mass, mitochondrial NO seems to be a chemical species located upstream in the signaling pathways that lead to the synthesis of new mitochondria in response to hyperglycemia.

In conclusion, complex I dysfunction and increased mitochondrial production rates of H_2O_2, NO, and $ONOO^-$ can be considered subcellular prodromal signals of the cardiac mitochondrial dysfunction that precedes myocardial failure in this type 1 DM model.

Conflicts of interest

None declared.

(See authors' conflicts of interest forms on the website/ Supplementary material)

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