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Diabetes mellitus reduces the function and expression of ATP-dependent K^+ channels in cardiac mitochondria

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ABSTRACT

Aim: Our goal was to determine the effects of type I diabetes mellitus on the function and expression of ATP-dependent K^+ channels in cardiac mitochondria (mitoK$_{ATP}$), composed of a pore-forming subunit (Kir6.1) and a diazoxide-sensitive sulphonylurea receptor (SUR1). We tested the hypothesis that diabetes reduces Kir6.1 and SUR1 expression as well as diazoxide-induced depolarization of mitochondrial membrane potential ($\Delta\Psi m$).

Main methods: Male FVB mice were made diabetic for 5 weeks with multiple low dose injections of streptozotocin. Cardiac mitochondria were separated into two populations: subsarcolemmal mitochondria (SSM) and interfibrillar mitochondria (IFM). mitoK$_{ATP}$ expression was determined via Western blot analysis of Kir6.1 and SUR1 proteins. mitoK$_{ATP}$ function was determined by measuring $\Delta\Psi m$ with the potentiometric dye rhodamine 123.

Key findings: Diabetes reduced Kir6.1 and SUR1 expression in IFM by over 40% ($p<0.05$ for both). Similarly, diabetes reduced Kir6.1 expression in SSM by approximately 40% ($p<0.05$); however, SUR1 expression was unaffected. Opening mitoK$_{ATP}$ with diazoxide (100 μM) depolarized control IFM $\Delta\Psi m$ by 80% of the valinomycin maximum; diabetic IFM depolarized only 30% ($p<0.05$). Diazoxide-induced depolarization was much less in SSM (20–30%) and unaffected by diabetes.

Significance: Our data indicate that diabetes reduces mitoK$_{ATP}$ expression and function in IFM. These changes in mitoK$_{ATP}$ may provide an opportunity to understand mechanisms leading to diabetic cardiomyopathy and loss of cardioprotective mechanisms in the diabetic heart.

Introduction

Mitochondrial ATP-dependent K^+ channels (mitoK$_{ATP}$) were first described in the liver (Inoue et al., 1991), but soon found in cardiac myocytes (Paucek et al., 1992), where they have been suggested to function in organellar volume regulation, apoptosis, and ischemic preconditioning (Ardehali and O'Rourke, 2005; Garlid et al., 2003; Hanley and Daut, 2005). Regarding the latter, cardioprotective roles for mitoK$_{ATP}$ have been shown in numerous studies (Garlid et al., 1997; Holmuhamedov et al., 1999; Queliconi et al., 2011). A great deal of interest surrounds therapeutic targets that could protect the heart against ischemia–reperfusion injury, including mitoK$_{ATP}$. In order to manipulate mitoK$_{ATP}$ function, it is important to consider its molecular composition. Functional analysis of mitoK$_{ATP}$ indicates that it is composed of Kir6.1 (an inwardly rectifying, K^+-selective pore) and SUR1 (Liu et al., 2001), a diazoxide-sensitive sulphonylurea receptor (Gopalakrishnan et al., 2000).

Mitochondrial dysfunction contributes to diabetic cardiomyopathy (Scheuermann-Freestone et al., 2003; Shen et al., 2004), but many of the mitochondrial mechanisms made aberrant by diabetes mellitus remain to be identified. Myocardial ischemic preconditioning is attenuated by diabetes mellitus (Hassouna et al., 2006; Katakam et al., 2007; Kersten et al., 2001), raising the possibility that diabetes mellitus may reduce the function and/or expression of mitoK$_{ATP}$ channels. However, the function and expression of mitoK$_{ATP}$ in the diabetic heart has not been examined directly. Thus, we tested the hypothesis that diabetes mellitus reduces Kir6.1 and SUR1 expression as well as diazoxide-induced depolarization of mitochondrial membrane potential ($\Delta\Psi m$).

However, in order to study mitoK$_{ATP}$ in the heart, one must consider that myocardial mitochondria exist in spatially-distinct subcellular regions and do not belong, necessarily, to a single homogenous population. Rather, two spatially-distinct subpopulations exist displaying different anatomy, biochemistry, and function (Hoppel et al., 2009; Weinstein et al., 1986). These subpopulations are referred to as interfibrillar mitochondria (IFM) and subsarcolemmal mitochondria (SSM). We have shown previously that type I diabetes mellitus more severely reduced IFM proteomic make-up as compared to SSM (Baseler et al., 2011; Dabkowski et al., 2009). Whether these

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observations extend to mitoK_{ATP} subunits remains to be determined, but such a decrease would be expected to also reduce mitoK_{ATP} function. To investigate our hypothesis, experiments were performed on isolated IFM and SSM of control and type I diabetic mice.

Methods

Animal model

Animal experiments conformed to the National Institutes of Health (NIH) *Guidelines for the Care and Use of Laboratory Animals* and were approved by our institutional Animal Care and Use Committee. Male FVB mice were housed in normal, bedded cages with access to food and water ad libitum. Eight-week-old mice, weighing at least 20 g, were randomly assigned to either a control or a diabetic group. Those in the diabetic group were given multiple low dose injections of streptozotocin (Leiter, 1982); a method we have used previously (Baseler et al., 2011; Dabkowski et al., 2010). On 5 consecutive days, mice were fasted for 6 h and then injected i.p. with 50 mg/kg streptozotocin (Sigma-Aldrich; St. Louis, MO). Dextrose water was provided ad libitum with the exception of fasting hours. Diabetes was confirmed by hyperglycemia (blood glucose ≥ 250 mg/dL). After 5 weeks of established hyperglycemia, mice were euthanized by CO_2 asphyxiation and the hearts excised for study.

Mitochondrial subpopulations

SSM and IFM were isolated by methods described previously (Palmer et al., 1977) with minor modifications (Baseler et al., 2011). Hearts were rinsed in cold phosphate buffered saline, blotted dry, and weighed. The ventricles were minced and homogenized 1:10 (w/v) in cold buffer containing (mM) 100 KCl, 40 Tris HCl, 10 Tris–base, 5 $MgCl_2$, 1 ATP, and 1 EDTA; pH 7.4. Homogenates were centrifuged at 800 ×*g* for 10 min to pellet the IFM entrapped in myofibrils (this is called spin 1 and referred to below). The supernatant of spin 1, which contains free-floating SSM, was collected and centrifuged at 9000 ×*g* to pellet the SSM. The SSM pellet was washed and centrifuged twice more at 9000 ×*g* to remove other cytosolic components. At this point, SSM are ready to use for Western blot or ΔΨm experiments (below).

The IFM/myofibril pellet from spin 1 was resuspended in buffer containing (mM) 100 KCl, 5 $MgSO_4$, 5 EGTA, and 50 Tris HCl; pH 7.4. IFM were liberated from the IFM/myofibril pellet by digesting with 0.5% trypsin at room temperature for 10 min. This digest was diluted two-fold with buffer and spun down at 5000 ×*g* for 5 min to pellet the IFM; the supernatant was discarded. The IFM pellet was resuspended then centrifuged at 800 ×*g* for 10 minutes. This supernatant, containing free-floating IFM, was collected. The cycle of fast and slow spins with washes was repeated to increase IFM yield. Finally, the combined supernatants, containing free-floating IFM, were centrifuged at 9000 ×*g* for 10 min to obtain a pellet of IFM. The supernatant was discarded and IFM were washed and pelleted two times to remove other components. At this point, IFM are ready to use for Western blot or ΔΨm experiments.

SSM and IFM from control and diabetic mouse hearts were used for Western blot and ΔΨm experiments. Our previous studies show that SSM and IFM populations prepared in this manner have different sizes, internal complexities, and proteomes (Baseler et al., 2011; Dabkowski et al., 2009, 2010). For Western blot analysis, IFM and SSM pellets were treated with 1% CHAPS and lysates stored at -80 °C until further use. When IFM and SSM were used for ΔΨm experiments, the pellets were resuspended in a buffer containing (mM) 120 KCl, 5 MOPS, 1 $MgSO_4$, and 1 EGTA; pH 7.4. This buffer was supplemented with 100 μM oligomycin to induce State 4 respiration. Mitochondria were stored on ice and used within 4 h.

Western blot

Mitochondrial protein (20 μg) samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 4–12% gels (Life Science Technologies; Grand Island, NY). Proteins were transferred to polyvinylidene fluoride (PVDF) membranes and blocked in 5% milk. Membranes were then probed overnight with rabbit primary antibodies to Kir6.1 (48 kDa; Alomone Labs; Jerusalem, Israel) or SUR1 (70 kDa; Millipore; Billerica, MA). To ensure equal protein loading and transfer efficiency, Kir6.1 and SUR1 expression were normalized to cytochrome *c* oxidase (COX IV; 16 kDa; Abcam; Cambridge, MA). Additionally, we used antibodies against the Na^+/K^+-ATPase (100 kDa; Santa Cruz Biotechnology; Santa Cruz, CA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 37 kDa; Sigma-Aldrich), and the voltage-dependent anion channel (VDAC; 30 kDa; Cell Signaling Technology; Danvers, MA) to test the quality of the IFM and SSM preparations. Membranes were incubated with secondary antibodies (BioRad; Hercules, CA) and chemiluminescence signals (GE Healthcare; Waukesha, WI) captured using a G:Box and GeneSnap software (Syngene; Frederick, MD).

ΔΨm measurements

Rho123 (λ_{ex} 503 nm, λ_{em} 527 nm) was used to assess ΔΨm as described previously (Aggarwal et al., 2010; Emaus et al., 1986) with a few modifications. Briefly, 0.3 mg/ml protein of intact IFM or SSM was loaded into a cuvette containing 5 nM Rho123 in respiration buffer composed of (mM) 120 KCl, 4 KH_2PO_4, 10 HEPES, 2 $MgCl_2$, and 0.1 EGTA; pH 7.25 (Baracca et al., 2003). This buffer also contained 5 mM pyruvate, 5 mM malate, 200 μM ADP, and 0.05 mg/ml bovine serum albumin. Mitochondrial uptake of Rho123 was allowed to stabilize before the addition of 100 μM diazoxide. Diazoxide effects are expressed as a percent of the maximum (1 μM) valinomycin-induced depolarization. A fluorometer (Turner Designs model 450; Sunnyvale, CA) and AxoScope software (Molecular Devices; Sunnyvale, CA) were used for data acquisition.

Statistical analysis

Data are shown as mean ± standard error of n number of animals. Comparisons between control and diabetic groups were made with unpaired t-tests. Statistical significance was set at $p<0.05$.

Results

Mice randomized to the control (n = 10) and diabetic (n = 10) groups weighed 22.4 ± 0.3 vs. 21.6 ± 0.4 g (p = NS) prior to the study. Mice injected with streptozotocin demonstrated a blood glucose concentration ≥ 250 mg/dL for inclusion in the diabetic group. At the time of sacrifice, mice in the control and diabetic groups weighed 26.4 ± 0.6 vs. 25.4 ± 0.4 g (p = NS). Neither heart weights (103.0 ± 4.4 vs. 99.7 ± 1.9 mg) nor heart weight to body weight ratios

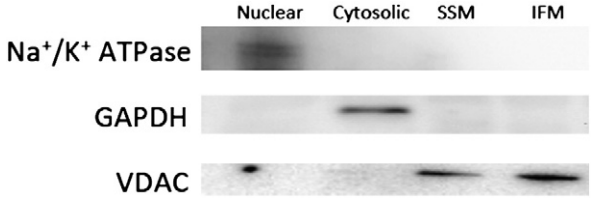

Fig. 1. Isolation of cardiac mitochondrial subpopulations. IFM and SSM are devoid of nuclear and cytosolic contamination. Na^+/K^+ ATPase immunoreactivity (100 kDa) is present in the nuclear/membrane fraction. GAPDH immunoreactivity (37 kDa) is present only in the cytosolic fraction. SSM and IFM fractions exclusively demonstrate VDAC immunoreactivity (30 kDa).