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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 μ M) 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.

Keywords

Kir6.1; SUR1; mitoK_{ATP}; membrane potential; diazoxide interfibrillar; subsarcolemmal

Introduction

Mitochondrial ATP-dependent K⁺ channels (mitoK_{ATP}) were first described in 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).

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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. 2010). 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 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 hr 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 CO₂ 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 MgCl₂, 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 $9,000 \times g$ to pellet the SSM. The SSM pellet was washed and centrifuged twice more at $9,000 \times g$ to remove other cytosolic components. At this point, SSM are ready to use for Western blot or $\Delta\psi_m$ experiments (below). The IFM/myofibril pellet from spin 1 was resuspended in buffer containing (mM) 100 KCl, 5 MgSO₄, 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 $5,000 \times g$ for 5 min to pellet the IFM; the supernatant was discarded. The IFM pellet was resuspended then centrifuged at $800 \times g$ 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 $9,000 \times g$ for 10 min to obtain a pellet of IFM. The supernatant was discarded and IFM washed and pelleted two times to remove other components. At this point, IFM are ready to use for Western blot or $\Delta\psi_m$ experiments.

SSM and IFM from control and diabetic mouse hearts were used for Western blot and $\Delta\psi_m$ 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 $\Delta\psi_m$ experiments, the pellets were resuspended in a buffer containing (mM) 120 KCl, 5 MOPS, 1 MgSO₄, 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 hr.

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).

$\Delta\psi_m$ measurements

Rho123 (λ_{ex} 503 nm, λ_{em} 527 nm) was used to assess $\Delta\psi_m$ 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 KH₂PO₄, 10 HEPES, 2 MgCl₂, 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 $\pm$ 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 ($p = \text{NS}$) 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 ($p = \text{NS}$). Neither heart weights (103.0 ± 4.4 vs. 99.7 ± 1.9 mg) nor heart weight to body weight ratios (3.89 ± 0.09 vs. 3.92 ± 0.04 mg/g) were different between groups. We have previously demonstrated diabetic cardiomyopathy in this model including decreased inotropy and lusitropy (Dabkowski et al. 2009).

The SSM and IFM preparations were devoid of sarcolemmal contamination, as immunoreactivity for the Na^+/K^+ -ATPase, an integral membrane protein, was restricted to the nuclear/membrane fraction after differential centrifugation (Fig. 1). Similarly, cytosolic constituents did not contaminate the SSM and IFM preparations, as immunoreactivity for GAPDH, a cytosolic enzyme, was restricted to the cytosolic fraction (Fig. 1). Immunoreactivity for VDAC of the mitochondrial outer membrane was restricted to the SSM and IFM preparations (Fig. 1).

SSM and IFM from control and diabetic mice were examined for Kir6.1 and SUR1 expression via Western blot analysis (Figs. 2A and 3A). Expression of Kir6.1, the pore forming subunit of $\text{mitoK}_{\text{ATP}}$, was reduced 41% in the SSM of diabetic mice ($p < 0.05$; Fig. 2B). Diabetes mellitus had no effect on the expression of SUR1, the regulatory subunit of $\text{mitoK}_{\text{ATP}}$, in SSM (Fig. 2C). Diabetes mellitus reduced Kir6.1 expression 45% in IFM ($p < 0.05$; Fig. 3B). Similarly, diabetes mellitus reduced SUR1 expression 42% in IFM ($p < 0.05$; Fig. 3C).

The potentiometric dye Rho123 was used to determine $\Delta\psi_{\text{m}}$ in SSM and IFM from control and diabetic mice (Figs. 4 and 5). Diazoxide, a SUR1-specific $\text{mitoK}_{\text{ATP}}$ agonist, was used to depolarize $\Delta\psi_{\text{m}}$; effects of diazoxide on $\Delta\psi_{\text{m}}$ were expressed as a percentage of the maximum valinomycin-induced depolarization. Diazoxide ($100 \mu\text{M}$) had a small effect on $\Delta\psi_{\text{m}}$ in SSM (Fig. 4). Further, there was no difference between SSM from control and diabetic mice (Fig. 4C). Diazoxide depolarized $\Delta\psi_{\text{m}}$ significantly in IFM from control mice (Fig. 5A). In contrast, diazoxide had little effect on $\Delta\psi_{\text{m}}$ in IFM from diabetic mice (Fig. 5B). Specifically, diabetes mellitus reduced diazoxide-induced depolarization 62% in IFM ($p < 0.05$; Fig. 5C).

Discussion

The incidence of diabetic cardiomyopathy, described as heart failure independent of coronary artery disease and hypertension (Regan et al. 1977; Rubler et al. 1972), is increasing (de Simone et al. 2010). We and others have shown that mitochondrial dysfunction contributes to diabetic cardiomyopathy (Baseler et al. 2011; Dabkowski et al. 2009, 2010; Scheuermann-Freestone et al. 2003; Shen et al. 2004; Williamson et al. 2010). However, many mitochondrial mechanisms made dysfunctional by diabetes mellitus remain

to be determined. For example, mitoK_{ATP} channels are important to mitochondrial function (Ardehali and O'Rourke 2005; Garlid et al. 2003; Hanley and Daut 2005), but the expression and function of mitoK_{ATP} channels in the diabetic heart has not been investigated thoroughly. These mitoK_{ATP} channels are likely composed of Kir6.1 and diazoxide-sensitive SUR1 (Gopalakrishnan et al. 2000; Liu et al. 2001); therefore, we tested the hypothesis that diabetes reduces Kir6.1 and SUR1 expression as well as diazoxide-induced depolarization of $\Delta\psi_m$. That is, we predicted diabetes mellitus would reduce the expression and function of mitoK_{ATP} in cardiac mitochondria.

Our data support the idea that type I diabetes mellitus reduces mitoK_{ATP} expression and function. This meshes well with reports that the diabetic heart is resistant to ischemic preconditioning (Hassouna et al. 2006; Katakam et al. 2007; Kersten et al. 2001), as this is a response mediated, at least in part, by mitoK_{ATP} channels (Garlid et al. 1997; Holmuhamedov et al. 1999; Queliconi et al. 2010). This suggests that a diabetes-induced decrease in mitoK_{ATP} expression may explain, in part, the loss of preconditioning in response to volatile anesthetics like isoflurane (Kehl et al. 2002). Conversely, these data suggest that preventing changes in mitoK_{ATP} expression or augmenting mitoK_{ATP} function with pharmaceuticals may be useful in combating diabetic cardiomyopathy.

Type II diabetes is more prevalent than type I diabetes, but we studied mitoK_{ATP} function and expression in type I diabetes mellitus for a number of reasons. First, diabetic cardiomyopathy was originally described in patients with type I diabetes (Regan et al. 1977; Rubler et al. 1972), but subsequently described in patients with type II diabetes (Bouchard et al. 1989). Second, the reports regarding attenuation of ischemic preconditioning were first described in a model of type I diabetes (Kersten et al. 2001) and later in type II diabetes (Hassouna et al. 2006; Katakam et al. 2007). Third, a model of type I diabetes allowed us to study the effects of hyperglycemia without confounding factors associated with type II diabetes (e.g., obesity, hyperinsulinemia, hypertension). In this model of type I diabetes (Leiter 1982) multiple low dose injections of streptozotocin were administered. Previous work indicates that cardiac effects in this model of type I diabetes are ameliorated when mice receive insulin (Ramanadham et al. 1987), indicating that hyperglycemia, not streptozotocin per se, is the underlying cause of deleterious effects. Because of the prevalence and burden of type II diabetes mellitus, one of our future research goals is to further examine this mitoK_{ATP} hypothesis in an appropriate model such as the db/db mouse.

There is nearly universal agreement that K_{ATP}-like channels exist in mitochondria (Inoue et al. 1991; Pucek et al. 1992); however, the molecular composition of mitoK_{ATP} channels has been debated (Ardehali et al. 2004). Convincing data suggest that mitoK_{ATP} may be composed of Kir6.1 and SUR1 (Liu et al. 2001), but proteomic analysis of mitochondrial Kir6.1 immunoprecipitates revealed no evidence of this pore-forming subunit (Foster et al. 2008). Recently, evidence has emerged supporting the renal outer medullary K⁺ (ROMK) channel as an ATP- and diazoxide-sensitive channel in mitochondria (Foster et al. 2012). Additional research will be required to definitively identify molecular components of mitoK_{ATP}. Further, evidence suggests that there may be differences between IFM and SSM. Specifically, IFM have been reported to lack SUR1, as only SSM were shown to be diazoxide-sensitive (Rottlaender et al. 2010). Our data contradict this; however, it is worth noting that the involvement of mitoK_{ATP} in diazoxide-induced depolarization may depend upon the bioenergetic state (Aggarwal et al. 2010; Holmuhamedov et al. 2012). We observed robust diazoxide-induced depolarization of $\Delta\psi_m$ in IFM, but not SSM, that was attenuated by type I diabetes.

While our results support the hypothesis, they raise questions about how type I diabetes mellitus differentially affects mitoK_{ATP} expression and function in IFM and SSM. However,

differences in the degree to which type I diabetes reduces mitoK_{ATP} expression in IFM and SSM are not entirely unexpected, as we have shown previously that type I diabetes mellitus differentially affects the proteomes of these cardiac mitochondrial subpopulations (Baseler et al. 2011). It is unlikely that differences in gene expression can explain why mitoK_{ATP} subunits are differentially affected in IFM and SSM. This is because neither Kir6.1 nor SUR1 is encoded by mitochondrial DNA; therefore, transcriptional regulation occurs in the nucleus. As such, changes in transcription would be expected to change IFM and SSM mitoK_{ATP} subunit expression in parallel, but this was not observed. The same arguments apply to translational regulation. Translation of mitoK_{ATP} subunits also occurs outside of the mitochondrion and changes in translation would be expected to affect IFM and SSM equally. It is likely that type I diabetes mellitus differentially impacts mitoK_{ATP} protein import into IFM and SSM. Specifically, we have demonstrated previously that the import of a number proteins into IFM was more severely impacted by type I diabetes (Baseler et al. 2011). We cannot, however, exclude the possibility that the rates of mitoK_{ATP} subunit turnover or degradation might be different in IFM and SSM. Regardless of the specific mechanisms involved, our data support the idea that type I diabetes mellitus reduces mitoK_{ATP} expression and function in IFM. Whether such changes are observed in type II diabetes remains to be determined.

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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).

Fig. 2. Diabetes mellitus reduces Kir6.1 expression in SSM

(A) Kir6.1 immunoreactivity (48 kDa) is reduced in SSM from hearts of diabetic mice ($n = 5$ mice in each group). (B) SUR1 immunoreactivity (70 kDa) in SSM is unaffected by diabetes mellitus ($n = 6$ mice for both). The expression of mitoKATP subunits, Kir6.1 and SUR1, is normalized to COX IV (16 kDa) to account any for any possible disparities in protein loading or transfer efficiency. Asterisk indicates $p < 0.05$ vs. control by unpaired t-test.

Fig. 3. Diabetes mellitus reduces mitoK_{ATP} subunit expression in IFM

(A) Kir6.1 immunoreactivity (48 kDa) is reduced in IFM from hearts of diabetic mice (n = 5–6 mice in each group). (B) SUR1 immunoreactivity (70 kDa) is reduced in IFM from hearts of diabetic mice (n = 5–6 mice for each group). mitoK_{ATP} subunit expression is normalized to COX IV (16 kDa) to ensure equal protein loading and transfer efficiency. Asterisks indicate $p < 0.05$ vs. control by unpaired t-test.

Fig. 4. $\Delta\psi_m$ in SSM has little sensitivity to diazoxide

(A) Trace showing Rho123 (5 nM; Rho) uptake by SSM from a control mouse, little effect of diazoxide (100 μM; DZX), and valinomycin-induced depolarization (1 μM; Val). (B) Trace from diabetic SSM. (C) Group data for control (n = 4) and diabetic (n = 3) mice. Diazoxide had little effect on $\Delta\psi_m$ in SSM from either control or diabetic mice.

Fig. 5. Diabetes mellitus reduced mitoK_{ATP}-induced depolarization of IFM

(A) Trace showing Rho123 (5 nM; Rho) uptake by IFM from a control mouse, significant depolarization by diazoxide (100 μ M; DZX), and little further depolarization by valinomycin (1 μ M; Val). (B) Trace from diabetic IFM. Diazoxide had little effect on $\Delta\psi_m$ in IFM of diabetic mice. (C) Group data for control (n = 4) and diabetic (n = 7) mice. Asterisk indicates $p < 0.05$ vs. control by unpaired t-test