Enhanced apoptotic propensity in diabetic cardiac mitochondria: influence of subcellular spatial location


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Williamson CL, Dabkowski ER, Baseler WA, Croston TL, Alway SE, Hollander JM. Enhanced apoptotic propensity in diabetic cardiac mitochondria: influence of subcellular spatial location. Am J Physiol Heart Circ Physiol 298: H633–H642, 2010. First published December 4, 2009; doi:10.1152/ajpheart.00668.2009.—Cardiovascular complications, such as diabetic cardiomyopathy, account for the majority of deaths associated with diabetes mellitus. Mitochondria are particularly susceptible to the damaging effects of diabetes mellitus and have been implicated in the pathogenesis of diabetic cardiomyopathy. Cardiac mitochondria consist of two spatially distinct subpopulations, termed subsarcolemmal mitochondria (SSM) and interfibrillar mitochondria (IFM). The goal of this study was to determine whether subcellular spatial location is associated with apoptotic propensity of cardiac mitochondrial subpopulations during diabetic insult. Swiss Webster mice were subjected to intraperitoneal injection of streptozotocin or citrate saline vehicle. Ten weeks following injection, diabetic hearts displayed increased caspase-3 and caspase-9 activities, indicating enhanced apoptotic signaling ($P < 0.05$, for both). Mitochondrial size (forward scatter) and internal complexity (side scatter) were decreased in diabetic IFM ($P < 0.05$, for both) but not in diabetic SSM. Mitochondrial membrane potential ($\Delta\psi_m$) was lower in diabetic IFM ($P < 0.01$) but not in diabetic SSM. Mitochondrial permeability transition pore (mPTP) opening was increased in diabetic compared with control IFM ($P < 0.05$), whereas no differences were observed in diabetic compared with control SSM. Examination of mPTP constituents revealed increases in cyclophilin D in diabetic IFM. Furthermore, diabetic IFM possessed lower cytochrome $c$ and Bcl-2 levels and increased Bax levels ($P < 0.05$, for all 3). No significant changes in these proteins were observed in diabetic SSM compared with control. These results indicate that diabetes mellitus is associated with an enhanced apoptotic propensity in IFM, suggesting a differential apoptotic susceptibility of distinct mitochondrial subpopulations based upon subcellular location.

Diabetes

Myocardial cell death is a major determinant of cardiac outcome during pathological conditions, and as a result it is a key player in the pathogenesis of diabetic cardiomyopathy. Myocyte cell death results from both necrotic and apoptotic mechanisms, both of which have been reported to be increased in the hearts of patients with diabetes (17, 18). It has been suggested that apoptotic cell death associated with diabetic cardiomyopathy is increased to a greater extent than necrotic cell death (18). Increases in apoptosis are not limited to human studies and have been reported in the hearts of diabetic animals (2, 5, 16, 19), as well as in cell culture systems utilizing high glucose media conditions representative of the diabetic state (17, 44). A number of markers have been used to determine increased apoptosis in the diabetic heart including terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL), cytosolic cytochrome $c$ content, and caspase activation (5, 7, 19, 44). Cellular apoptosis can occur through a number of different mechanisms, affecting distinct apoptotic cascades although it is not entirely clear whether multiple apoptotic cascades are involved in diabetic cardiomyopathy, since results are varied between studies (5, 19). Mitochondrion-dependent apoptotic mechanisms have been shown to be increased during diabetic insult and have been correlated with the onset of diabetic cardiomyopathy (5, 8, 19). Activation of caspase-3 in response to enhanced cytosolic cytochrome $c$ was reported in H9c2 cells exposed to hyperglycemia, suggesting that increased glucose presence is at least partly responsible for the enhanced apoptosis observed during diabetes. Furthermore, these authors suggest that reactive oxygen species (ROS) derived from high glucose levels may trigger apoptosis, implicating a link between ROS generation and the apoptotic program (8). These results are in agreement with others indicating increased mitochondrial permeability transition pore (mPTP) opening resulting from high glucose incubation conditions (44).

Cardiac mitochondria exist in distinct subcellular spatial arrangements. Two spatially distinct mitochondrial populations have been identified and termed subsarcolemmal mitochondria (SSM) and interfibrillar mitochondria (IFM) due to their subcellular locations, in which either exist beneath the sarcolemma or situate between the contractile apparatus (34, 37). In addition to differences in subcellular locality, mitochondrial subpopulations differ in a number of other structural, morphological, and biochemical properties, which have been characterized by others (1, 21, 24, 25, 27, 29, 32, 34). As a result of their diverse properties, spatially distinct mitochondrial subpopulations respond differently to physiological stimuli, including exercise, aging, obesity, fasting, and ischemia-reperfusion injury (1, 10, 27, 29, 32, 36, 39). Recently, our laboratory observed differential responses of spatially distinct mitochondrial subpopulations during streptozotocin (STZ)-induced diabetic insult in a mouse model (9). Others have reported differential responses of cardiac mitochondrial subpopulations in a rat model, using STZ treatment (26). These results suggest that although mitochondria are similar in their central role in cellular function, spatially distinct subpopulations are influenced by pathological states differently, requiring thorough examination of individual mitochondrial subpopulations during disease states.

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Previous findings from our laboratory indicate that in terms of morphology, metabolic function, and oxidative stress, spatially distinct mitochondrial subpopulations respond differently to STZ-induced diabetic insult, with the IFM subpopulation being most affected (9). Because previous studies examining the impact of diabetes mellitus on apoptotic propensity have been performed on total mitochondria, we sought to determine the differential response of individual mitochondrial subpopulations subjected to a diabetic phenotype, in an effort to understand their specific contributions to enhanced apoptosis associated with diabetic cardiomyopathy. Our findings suggest that the IFM subpopulation has a greater propensity to undergo apoptosis during STZ-induced diabetes mellitus compared with the SSM. This study is the first to determine the subcellular spatial influence of the diabetic phenotype on mitochondrially associated apoptosis.

MATERIALS AND METHODS

Experimental animals and diabetes induction. The animal experiments in this study conformed to the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals and were approved by the West Virginia University Animal Care and Use Committee. Female Swiss Webster mice (Harlan, Indianapolis, IN) were housed in the West Virginia University Health Sciences Center animal facility. Mice were given unlimited access to a rodent diet and water. Diabetes was induced in 8-wk-old mice (20–25 g) as previously described (41), using a single high-dose STZ (Sigma, St. Louis, MO) injection. Intraperitoneal injection of 175 mg/kg body wt STZ, dissolved in sodium citrate buffer (pH 4.5), was performed following 12 h of fasting. Mice that served as vehicle controls were given the same volume per body weight of sodium citrate buffer. One week before diabetes induction, animals were euthanized and the atria following the onset of hyperglycemia, the animals were euthanized and weighed. SSM and IFM were separately homogenized in 12 mg/g of trypsin for 10 min. After 10 min, the IFM pellet was resuspended in KME buffer containing (in mmol/l) 100 mM KCl, 50 mM MOPS, 5 mM MgSO4, 7H2O, 1 mM EGTA, and 1 mM ATP (pH 7.4 at 4°C). The homogenates were centrifuged at 700 g for 10 min. The supernatant containing the SSM was extracted and centrifuged at 10,000 g for 10 min. The pellet was washed and centrifuged two more times at 10,000 g for 10 min. The remaining pellet from the 700 g spin was resuspended in KME buffer containing (in mmol/l) 100 mM KCl, 50 mM MOPS, and 0.5 mM EGTA (pH 7.4) and incubated with 5 mg/g of trypsin for 10 min. After 10 min, the IFM pellet was diluted twofold with buffer and centrifuged at 700 g for 10 min. The IFM containing supernatant was then centrifuged at 10,000 g for 10 min and combined with the first supernatant to maximize IFM yield. The combined supernatant was centrifuged at 10,000 g for 10 min, and the pellet was resuspended again and subjected to two more washes and centrifuged at 10,000 g for 10 min. Citrate synthase activity was examined using a commercially available kit (Sigma). Protein concentrations were determined as above.

Mitochondria size and internal complexity. To index mitochondrial subpopulation size and complexity, we performed flow cytometry analyses using a FACS Calibur equipped with a 15 mw 488 nm argon ion laser. For each activity assay, specific substrates were added, Ac-DEVD-AFC (Alexis Biochemicals, San Diego, CA) for caspase-3 activity and Ac-LEHD-AFC (Alexis Biochemicals) for caspase-9 activity, to 200 μg of sample and incubated at 37°C for 1 to 2 h. Samples were analyzed fluorometrically on a Biotek Synergy HT plate reader (Biotek, Winooski, VT) immediately and at 1 and 2 h after substrate addition. Fluorometric measurements were performed at 400 nm/505 nm and expressed per protein content. Protein concentrations were determined using the Bradford method and bovine serum albumin as a standard (6).

Preparation of individual mitochondrial subpopulations. Ten weeks following the onset of hyperglycemia, animals were euthanized and the atria was removed. Ventricular tissue was rinsed in phosphate-buffered saline (PBS; pH 7.4) and then blotted dry and weighed. SSM and IFM were isolated on ice following the methods of Palmer et al. (34), with minor modifications (9, 10). Briefly, the ventricles were minced and homogenized 1:10 (wt/vol) in cold Chappel-Perry buffer containing (in mmol/l) 100 mM KCl, 50 mM MOPS, 5 mM MgSO4, 7H2O, 1 mM EGTA, and 1 mM ATP (pH 7.4 at 4°C). The homogenates were centrifuged at 700 g for 10 min. The supernatant containing the SSM was then centrifuged at 10,000 g for 10 min. The pellet was washed and centrifuged two more times at 10,000 g for 10 min. The remaining pellet from the 700 g spin was resuspended in KME buffer containing (in mmol/l) 100 mM KCl, 50 mM MOPS, and 0.5 mM EGTA (pH 7.4) and incubated with 5 mg/g of trypsin for 10 min. After 10 min, the IFM pellet was diluted twofold with buffer and centrifuged at 700 g for 10 min. The IFM containing supernatant was then centrifuged at 10,000 g for 10 min and combined with the first supernatant to maximize IFM yield. The combined supernatant was then centrifuged at 10,000 g for 10 min, and the pellet was resuspended again and subjected to two more washes and centrifuged at 10,000 g for 10 min. Citrate synthase activity was examined using a commercially available kit (Sigma). Protein concentrations were determined as above.

Mitochondrial apoptosis and diabetic cardiomyopathy

Table 1. Heart weight, body weight, and mitochondrial yields

<table>
<thead>
<tr>
<th>Body Weight, g</th>
<th>Heart Weight, mg</th>
<th>Heart Weight to Body Weight, mg/g</th>
<th>Subsarcolemmal Mitochondria Protein, mg/g tissue</th>
<th>Interfibrillar Mitochondria Protein, mg/g tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>30±1</td>
<td>113±3</td>
<td>3.8±0.08</td>
<td>10.97±0.94</td>
</tr>
<tr>
<td>Diabetic</td>
<td>24±1*</td>
<td>95±7*</td>
<td>3.8±0.14</td>
<td>12.84±2.11</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 8 for each group. Heart weight, body weight, and heart weight-to-body weight ratios 10 wk following streptozotocin treatment (diabetic) or citrate saline injection (control) are shown. *P < 0.05 for diabetic vs. control.
laser and 633 red diode laser (Becton and Dickinson, San Jose, CA) as previously described (9, 10). Each individual parameter (gating, size, and complexity) was performed using specific light sources (laser, photomultiplier tube) and specific detectors. MitoTracker Deep Red 633 (Invitrogen, Carlsbad, CA), which passively diffuses into intact mitochondria due to membrane potential, was used to selectively stain intact mitochondria (emission, 633 nm; fluorescent 633 red diode laser). This approach excludes debris, which contains no membrane potential, enabling accurate gating (R1) of the mitochondria. Once the gating parameters were established, gated events (20,000/sample) were subsequently examined using the forward scatter detector (FSC; 488 nm argon laser and diode detector) and side scatter detector (SSC; photomultiplier tube and 90° collection lens) and represented in FSC versus SSC density plots. Geometric mean (arbitrary units) representing FSC (logarithmic scale) was used as an indicator of size, whereas values from SSC (logarithmic scale) were used to indicate complexity. Although the FSC arbitrary unit is proportional to the individual mitochondria particle size, the absolute value still remains an arbitrary unit. All flow cytometric measurements were performed under the supervision of the West Virginia University Flow Cytometry Core Facility.

Mitochondrial membrane potential. Mitochondrial membrane potential \( (\Delta \psi_m) \) was measured by flow cytometry using the ratiometric dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol carbocya-

Fig. 2. Mitochondria subpopulation morphological assessment. Determination of relative size and internal complexity of distinct mitochondrial subpopulations using flow cytometric analyses is shown. A: representative density plot of isolated mitochondria stained with MitoTracker Deep Red 633. Intact mitochondria are gated (R1) and represented in green, whereas noise and debris (outside the R1 gate) are indicated below the green line. B: representative gated density plot from a control mouse indicating size (forward scatter (FSC)) versus internal complexity (side scatter (SSC)) of isolated subsarcolemmal mitochondria (SSM). C: representative gated density plot from a control mouse indicating size (FSC) versus internal complexity (SSC) of isolated interfibrillar mitochondria (IFM).

In each case, analysis of FSC and SSC was calculated per 20,000 gated events for all mitochondrial subpopulations. D: analysis of cardiac SSM and IFM size (FSC) in control and diabetic mitochondria subpopulations. E: analysis of cardiac SSM and IFM complexity (SSC) in control and diabetic mitochondria subpopulations. Values for FSC and SSC are expressed as arbitrary units (AU) ± SE; n = 4 for each group. *P < 0.05 for diabetic vs. control.
nine iodide (JC-1; Molecular Probes, Carlsbad, CA), which is a lipophilic cation that enters selectively into mitochondria. Isolated mitochondrial subpopulations were incubated with JC-1 for 15 min at 37°C, and 20,000 gated events were analyzed per sample. Changes in $\Delta\psi_m$ are reflected in the degree of color change from green to orange as membrane potential increases. The shift to orange is due to the dye forming aggregates upon membrane polarization causing shifts in emitted light from 530 nm (green) to 590 nm (orange). Addition of 200 $\mu$M of dinitrophenol, which collapses the $\Delta\psi_m$, was used as an assay control. Measurements were performed on freshly isolated mitochondria subpopulations. Values are expressed as the mean orange fluorescence divided by the mean green fluorescence of 20,000 mitochondrial events per individual mitochondrial sample.

$mPTP$ opening. mPTP opening propensity was determined spectrophotometrically (540 nm) by monitoring the decrease in light scattering associated with mitochondrial swelling as previously described (1, 22). Swelling was induced by treatment with 400 $\mu$M Ca$^{2+}$, 100 $\mu$M tert-butyl hydroperoxide ($\text{t-BuOOH}$), and 10 mM succinate and then followed for 15 min. Addition of 1 $\mu$M cyclosporin A (CSA), a specific mPTP inhibitor, was used as an assay control. As above, measurements were performed on freshly isolated mitochondria subpopulations.

$mPTP$ constituents. SDS-PAGE was run on 4–12% gradient gels as described with equal amounts of protein loaded for each study treatment (28). Relative amounts of subpopulation-specific mitochondrial cyclophilin D, adenine nucleotide translocase (ANT), and voltage-dependent anion channel (VDAC) were determined using specific antibodies: anti-cyclophilin D rabbit antibody (Product No. PA1-028; Affinity Bioreagents, Golden, CO), anti-ANT goat antibody (Product No. sc-9300; Santa Cruz Biotech, Santa Cruz, CA), and anti-VDAC rabbit antibody (Product No. 4866; Cell Signaling, Danvers, MA). The secondary antibody was a goat anti-rabbit IgG horseradish peroxidase (HRP) conjugate (Product No. 10004301; Cayman Chemical) for cyclophilin D and Bax and a goat anti-mouse IgG HRP conjugate for Bcl-2 (Product No. 31430; Pierce Biotechnology). Detection of signal was performed according to the manufacturer’s instructions, using Pierce ECL Western blotting substrate (Pierce). Autoradiographic signals were assessed, and the data were analyzed as above. Control for protein loading was confirmed by Coomassie blue staining, as above.

Statistics. Means and SE were calculated for all data sets. Data were analyzed with a Student’s $t$-test to evaluate mitochondrial subpopulation treatment effects (diabetes induction) using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). $P < 0.05$ was considered significant.

RESULTS

Diabetes-induced caspase activation. Heart weights and body weights were significantly decreased ($P < 0.05$, for both) following STZ treatment (Table 1). As a result, no differences were observed in heart weight-to-body weight ratios (Table 1). No significant differences were observed in subpopulation protein yields between control and diabetic mitochondria (Table 1). To determine whether there was increased apoptotic signaling associated with diabetic insult, we examined caspase-3 and -9 activities. Diabetic hearts had increased caspase-3 and -9 activities compared with control (Fig. 1). Caspase-3 activity was increased by 54% ($P < 0.05$) in the diabetic heart, whereas caspase-9 activity was increased by
37% ($P < 0.05$) in the diabetic heart. These results indicate that caspase-3 and -9 activities are increased in the heart during STZ-induced diabetic insult, reflective of enhanced apoptotic signaling.

**Mitochondrial subpopulation morphology.** We confirmed mitochondrial integrity following isolation by examining the enzymatic activity of citrate synthase. Assessment of citrate synthase activity revealed minimal activity in the supernatant fraction compared with the mitochondrial fraction ($<10\%$ for supernatant vs. $>90\%$ for mitochondria), with no significant differences between SSM and IFM or control and diabetic. To determine morphological differences between control and diabetic mitochondrial subpopulations, we used a flow cytometry approach. In Fig. 2A, a typical dot plot, showing MitoTracker Deep Red 633 stained mitochondria, is shown, with intact mitochondria indicated in green and unstained debris indicated below the green line. Using this information, we gated the mitochondria (R1) to exclude unstained debris and applied the R1 gate to analyses on the individual subpopulations. SSM were larger in size (FSC) and possessed greater internal complexity (SSC) compared with IFM, which were smaller and more compact (Fig. 2, B vs. C). These results are consistent with previous findings from our laboratory utilizing this methodology (9). In addition, these results are consistent with previously published reports using other methods for determining mitochondrial morphology (25, 36–38). Mitochondrial size (FSC) was significantly decreased by 14% ($P < 0.05$) in the diabetic compared with control IFM, whereas SSM showed no significant changes (Fig. 2D). Mitochondrial complexity (SSC) in the diabetic IFM was significantly decreased by 15% ($P < 0.05$), compared with control IFM (Fig. 2E). No significant differences in SSC were observed in the SSM population (Fig. 2E). These results are similar to previous findings from our laboratory, but the degree and extent of change were considerably different (9).

**Mitochondrial subpopulation membrane potential.** $\Delta V_m$ was analyzed in freshly isolated mitochondrial subpopulations by flow cytometry using JC-1. In Fig. 3A, a typical histogram shows the SSM and IFM stained with JC-1 dye. The shift from green to orange indicates an increase in $\Delta V_m$. Overall, $\Delta V_m$ was greater in IFM compared with SSM, which is in agreement with previous reports (37). SSM from control and diabetic hearts showed no difference in $\Delta V_m$ (Fig. 3, A and B). However, IFM $\Delta V_m$ was decreased by 54% ($P < 0.01$) in diabetic hearts compared with control (Fig. 3, A and B). These data suggest that IFM $\Delta V_m$ is decreased relative to control during diabetic insult, whereas SSM $\Delta V_m$ remains unaffected.

**mPTP subpopulation opening.** We determined whether diabetic insult differentially influences the susceptibility of mitochondrial subpopulations to undergo mitochondrially initiated apoptosis by assessing mPTP opening through examination of mitochondrial swelling associated with exogenous oxidant exposure. Pore opening is associated with expansion of the space occupied by the mitochondrial matrix, and the time to $V_{\text{max}}$ is representative of the pore opening rate (1, 22). In Fig. 4, A and B, representative absorbance plots are shown. Overall, control IFM had a greater time to $V_{\text{max}}$ than control SSM (Fig. 4B, solid black line vs. Fig. 4A, solid black line), which is in agreement with other reports (1). Addition of CsA, a pore opening inhibitor, increased times to $V_{\text{max}}$ (Fig. 4, A and B, solid and hatched gray lines). Time to $V_{\text{max}}$ was significantly decreased in diabetic compared with control IFM (Fig. 4B, hatched black line vs. solid black line, and 4C; $P < 0.05$). SSM time to $V_{\text{max}}$ remained unaffected by diabetic insult (Fig. 4A, solid black line vs. hatched black line, and 4C). These data suggest that diabetic IFM have lower resistance to oxidative-stress-induced pore opening compared with control IFM.

**mPTP subpopulation constituents.** We examined the protein levels of several mPTP proteins in individual mitochondrial subpopulations following diabetic insult. Specific pore components examined included cyclophilin D, ANT, and VDAC.

![Graph](http://ajpheart.physiology.org/)

**Fig. 4.** Mitochondria permeability transition pore (mPTP) subpopulation opening. mPTP opening assessed by spectrophotometric measurement at 540 nm of mitochondrial swelling following oxidant (400 μM r-BuOOH, Ca$^{2+}$ (100 μM), and succinate (10 mM) exposure are shown. Time to $V_{\text{max}}$ was assessed over a 15-min time period following exogenous oxidant exposure. $A$: representative absorbance plot. Control SSM, solid black line; diabetic SSM, hatched black line; control SSM + cyclosporin A (CsA), solid gray line; diabetic SSM + CsA, hatched gray line. $B$: representative absorbance plot. Control IFM, solid black line; diabetic IFM, hatched black line; control IFM + CsA, solid gray line; diabetic IFM + CsA, hatched gray line. $C$: graph of all samples examined. Values are expressed as means ± SE; $n = 6$ for each group. *$P < 0.05$ for diabetic vs. control.
results indicate that cyclophilin D is increased in diabetic compared with control IFM (Fig. 5B; P < 0.05). However, there was no difference in cyclophilin D in diabetic and control SSM (Fig. 5A). No significant differences were observed in ANT and VDAC contents in any group examined (Fig. 5, C–F). These results indicate that diabetic IFM have increased cyclophilin D protein levels compared with control IFM.

Specific mitochondrial subpopulation protein contents. To determine mechanisms influencing the enhanced apoptosis observed in the diabetic heart, we examined protein contents of Bax, and Bcl-2, and cytochrome c in spatially distinct mitochondrial subpopulations. Bax protein levels were increased in diabetic compared with control IFM (Fig. 6B; P < 0.05), yet no change was observed in SSM (Fig. 6A). Bcl-2 protein content was decreased in the diabetic IFM compared with control IFM (Fig. 6D; P < 0.05), but no change was observed in SSM (Fig. 6C). Mitochondrial cytochrome c content was decreased in the diabetic compared with control IFM (Fig. 7B; P < 0.05). However, no change was observed in cytochrome c content in the diabetic SSM compared with control (Fig. 7A). Additionally, an overall increase in cytosolic cytochrome c content was observed (Fig. 7C; P < 0.05). These results suggest that critical proteins involved in the apoptotic process are altered in response to diabetic insult specifically in the IFM.

Fig. 5. mPTP subpopulation components. mPTP components assessed by Western blot analyses. A: representative Western blots for cyclophilin D and summary data from control and diabetic SSM and IFM (B). C: representative Western blots for adenine nucleotide translocase and summary data from control and diabetic SSM and IFM (D). E: representative Western blots for voltage-dependent anion channel and summary data from control and diabetic SSM and IFM (F). Control for protein loading was confirmed by Coomassie blue staining. Values are expressed relative to control as means ± SE; n = 4 for each group. *P < 0.05 for diabetic vs. control.
A significant amount of data suggests the existence of a myocardial pathology associated with diabetes mellitus and existing independent of coronary artery disease, hypertension, or other cardiac abnormality (12, 45). Despite the growing evidence for the existence of a diabetic cardiomyopathy, the mechanisms involved in the pathogenesis are not entirely clear. In this study, we determined whether mitochondrial associated apoptotic propensity was subject to subcellular spatial influence during diabetic insult. The impetus for the proposed experiments was to build upon our previous study characterizing the differential response of spatially distinct mitochondrial subpopulations following STZ treatment by differentiating the relative propensity for mitochondria to undergo apoptosis in an effort to begin to incorporate subcellular spatial influence into the mechanistic pathogenesis of diabetic cardiomyopathy. For our experimentation, we used a well-established type 1 diabetes mellitus model STZ injection. Ten weeks following diabetes onset, we observed decreases in both heart and body weights of STZ-injected mice (Table 1). These results are in agreement with other studies utilizing a single high-dose STZ model (8, 44). Because our results reflect those changes observed in female mice, one needs to consider that sex differences may impart differential responses.

Enhanced apoptosis resulting from diabetic insult has been reported previously (2, 5, 7, 8, 19, 44). Cellular apoptosis is initiated by numerous pathological and physiological stimuli, which include receptor-mediated mechanisms as well as mitochondrial mechanisms. Central to the apoptotic process is the coordinated activation of the family of cysteine proteases caspases. In the current study, we determined whether STZ-induced diabetic insult enhanced apoptotic signaling by assessing the activation of two key caspases: caspase-9 and caspase-3. Caspase-9 is activated by Apaf-1, which associates with cytochrome c released from the mitochondrion. Our data indicate that STZ-induced diabetic insult enhances caspase-9 activation and the downstream protein caspase-3 (Fig. 1). These results are in agreement with other reports indicating increased caspase activation resulting from the diabetic phenotype (5, 8, 19). We recognize that caspase activation is an upstream signaling event that precedes cellular apoptosis. Nevertheless, our results suggest a general increase in the level of apoptotic signaling following STZ-induced diabetic insult.

The central postulate being tested in the current study centered on understanding whether subpopulations of mitochondria contribute differently to diabetes-associated apoptosis in the heart. These examinations were predicated on the idea that mitochondria existing in distinct subcellular spatial locations may be affected by diabetic insult differently due in part to variations in morphology, biochemistry, and proteomic makeup (9). To this end, we examined morphological differences following diabetic insult, between SSM, which are located beneath the plasma membrane, and IFM, located between the contractile apparatus (34). Analyses were performed using established methodology from our laboratory, which employs a flow cytometric approach (9, 10). Our results indicate that in
terms of mitochondrial size and internal complexity, diabetic insult influences only the IFM with both parameters being decreased (Fig. 2, D and E). Overall, these results are in agreement with a previous study from our laboratory making similar assessments 5 wk following multiple low-dose STZ treatment (9), as well as others examining total mitochondria from type 2 diabetic patients (40). Despite this similarity, the overall decreases in size and internal complexity observed in our previous study were much greater than those observed in the current study. It is not entirely clear why these changes in size and complexity were disparate and may be a function of several factors. First, diabetes mellitus was examined over different time frames in the two studies (5 vs. 10 wk), and thus the results represent distinct snapshots for the pathology. Second, the current study utilized a single high-dose STZ protocol, whereas our previous study utilized a multiple low-dose STZ protocol. In addition, one cannot rule out the possibility of a biphasic morphological response in which a large initial decrease in mitochondrial size and complexity precedes a more subtle increase in both parameters, such that the absolute differences are lessened. Such a phenomenon may indicate an increase in mitochondrial swelling. Regardless, the results are interesting and warrant further examination.

The main goal of the current study was to determine whether spatially distinct sets of mitochondria contribute differently to cellular apoptosis during STZ-induced diabetic insult. To lend insight into this question, we examined diabetes-induced changes in ΔΨm, which are an early event that can precede mitochonndrially initiated apoptosis. Our results indicate that ΔΨm is significantly decreased relative to control, only in the IFM subpopulation (Fig. 3, A and B). These findings are in agreement with others examining total mitochondria in both type 1 (14, 42) and type 2 (13) models of diabetes mellitus. Interestingly, ΔΨm values were higher in control IFM compared with control SSM, reflective perhaps of the biochemical differences between the two subpopulations, such as oxidative phosphorylation rates (37). Nevertheless, our results are in contrast with other reports indicating higher basal ΔΨm in the SSM population of rat skeletal muscle (1). It is not entirely clear as to the reason for the discrepancy, although it may be due in part to differences in tissue and species type, as well as assay conditions. Decreases in ΔΨm may influence mPTP formation and opening. Because the mPTP is responsible for initiation of the mitochondrially associated apoptotic cascade, we determined whether diabetic insult differentially influences the propensity for individual mitochondrial subpopulations to undergo apoptosis following exogenous oxidant exposure. Our results indicate that relative to respective control values, diabetic IFM display significantly faster time to Vmax upon exogenous oxidant exposure, suggesting enhanced mPTP opening relative to control (Fig. 4, A and B). In general, these results are in agreement with other studies examining total mitochondria from human microvascular endothelial cells subjected to elevated glucose concentrations (11) and STZ-treated rat liver (15). Furthermore, IFM from aged heart have been shown to possess enhanced susceptibility to pore opening compared with SSM (20). Ferreira et al. (15) have suggested that the phospholipid environment, and specifically cardiolipin content, is associated with the susceptibility of mPTP induction. These authors go on to propose that loss of cardiolipin content would be associated with increased induction of the mPTP (15).
Results from a previous study from our laboratory examining cardiolipin content following STZ-induced diabetic insult indicate that IFM have a decreased content of a major cardiolipin species (9). Such a result correlates with the findings from our current study indicating increased propensity for IFM to undergo mPTP opening following exogenous oxidant exposure and is in agreement with the proposition by Ferreira et al. (15). The functional relevance for an enhanced propensity of IFM to undergo apoptosis upon a triggering stimulus is that the cardiomyocyte may present regional subcellular differences in the sensitivity of mitochondria to potential apoptotic initiators such as enhanced oxidants, which our laboratory has previously reported (9). As a result, the IFM may play a more prominent role than the SSM in mediating mitochondrially initiated apoptosis, suggesting that this subfraction is critically important in regulating apoptosis-associated myocyte loss in the diabetic heart. Despite our findings, it is important to point out that the mPTP measurement employed in this study only evaluates the susceptibility of mitochondria to undergo apoptosis, thus direct measurement of mitochondria-associated apoptosis is not performed. Further studies are needed to fully appreciate the functional relevance of these changes to the pathogenesis of diabetic heart disease.

A number of specific proteins have been implicated in the dynamics of the mPTP, and they include cyclophilin D, ANT, and VDAC. Studies suggest that certain proteins are critical for either regulation or eventual assembly of an open pore complex (3, 4, 23, 43, 46). As a result, we determined whether STZ-induced diabetic insult was associated with changes in some of these proteins suggested to play a role in mPTP dynamics. Our results indicate that STZ-induced diabetic insult was associated with an increase in cyclophilin D in IFM, without a concomitant change in SSM (Fig. 5, A and B). Additionally, no significant changes as a result of STZ-induced diabetic insult were observed with either ANT or VDAC expression in either subpopulation (Fig. 5, C–F). These findings are in partial agreement with others reporting no changes in ANT activity of STZ-treated rats (31) as well as others reporting no change in ANT and VDAC of an aged rat heart (20). Our results are in agreement with others demonstrating increased Bax-to-Bcl-2 ratios (35) or decreased Bcl-2 (30) in total cardiac mitochondria from obese Zucker rats. Taken together, these results suggest that diabetic IFM are more sensitive to ROS-induced mPTP opening as a result of changes in a particular subset of proteins that influence the pro-/antiapoptotic balance within mitochondria.

In conclusion, we report that type 1 diabetic insult impacts the apoptotic process differently in spatially distinct mitochondrial subpopulations. Type 1 diabetic insult is associated with enhanced apoptotic propensity in the IFM, which may be the result of differences in the makeup of specific proteins that impact mPTP dynamics. These results suggest that the enhanced mitochondrially initiated apoptosis associated with type 1 diabetic insult may occur to a greater extent in the IFM subpopulation.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

REFERENCES


