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Increased propensity for cell death in diabetic human heart is mediated by mitochondrial-dependent pathways

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Anderson EJ, Rodriguez E, Anderson CA, Thayne K, Chitwood WR, Kypson AP. Increased propensity for cell death in diabetic human heart is mediated by mitochondrial-dependent pathways. Am J Physiol Heart Circ Physiol 300: H118-H124, 2011. First published November 12, 2010; doi:10.1152/ajpheart.00932.2010.-Progressive energy deficiency and loss of cardiomyocyte numbers are two prominent factors that lead to heart failure in experimental models. Signals that mediate cardiomyocyte cell death have been suggested to come from both extrinsic (e.g., cytokines) and intrinsic (e.g., mitochondria) sources, but the evidence supporting these mechanisms remains unclear, and virtually nonexistent in humans. In this study, we investigated the sensitivity of the mitochondrial permeability transition pore (mPTP) to calcium (Ca²⁺) using permeabilized myofibers of right atrium obtained from diabetic (n = 9) and nondiabetic (n = 12) patients with coronary artery disease undergoing nonemergent coronary revascularization surgery. Under conditions that mimic the energetic state of the heart in vivo (pyruvate, glutamate, malate, and 100 µM ADP), cardiac mitochondria from diabetic patients show an increased sensitivity to Ca2+-induced mPTP opening compared with nondiabetic patients. This increased mPTP Ca² sensitivity in diabetic heart mitochondria is accompanied by a substantially greater rate of mitochondrial H₂O₂ emission under identical conditions, despite no differences in respiratory capacity under these conditions or mitochondrial enzyme content. Activity of the intrinsic apoptosis pathway mediator caspase-9 was greater in diabetic atrial tissue, whereas activity of the extrinsic pathway mediator caspase-8 was unchanged between groups. Furthermore, caspase-3 activity was not significantly increased in diabetic atrial tissue. These data collectively suggest that the myocardium in diabetic patients has a greater overall propensity for mitochondrial-dependent cell death, possibly as a result of metabolic stress-imposed changes that have occurred within the mitochondria, rendering them more susceptible to insults such as Ca²⁺ overload. In addition, they lend further support to the notion that mitochondria represent a viable target for future therapies directed at ameliorating heart failure and other comorbidities that come with diabetes.

diabetes; mitochondria; apoptosis

THE PATHOGENIC MECHANISMS by which persistent hyperglycemia and dyslipidemia in diabetes adversely affect the coronary vasculature are well characterized (21), but the mechanisms by which diabetes creates a progressively degenerative state within the myocardium are not as clear. Recently, investigators have focused on the role of myocardial cell death because of the increasing number of studies demonstrating high levels of apoptotic and necrotic cardiomyocytes in experimental models of diabetes (4, 23, 27) and in cardiac tissue from diabetic patients (14, 25, 37). Signals initiating myocardial cell death originate from intrinsic (e.g., mitochondria) and extrinsic (e.g., neurohumoral factors) sources (30). It is not fully known whether and to what extent one pathway predominates over the other in diabetic cardiomyopathy, although studies using experimental models have recently shown that the mitochondrialdependent, intrinsic pathway may play a large role (27, 56).

Mitochondria coordinate cell death in the diseased myocardium via a number of different mechanisms, ranging in complexity and temporal activity. Cell necrosis, the most rapid form of death mediated by the opening of a Ca^{2+} -sensitive, multiprotein pore complex in the mitochondrial inner membrane (permeability transition pore, mPTP), has been demonstrated to be a causative factor in various cardiomyopathies (11, 43). The slower-acting, mitochondrial-mediated apoptosis involves a diverse and complex array of initiators and intermediates, ranges in time from hours to days, and has been implicated in many different cardiomyopathies.

Importantly, a role for mitochondrial-mediated apoptosis in the etiology of diabetic cardiomyopathy has been clearly established in experimental models (4, 12, 28, 52, 56), although evidence is lacking in humans. Moreover, much remains to be understood about the relationship between hyperglycemia and apoptosis in the diabetic heart. As in most diabetic-related complications, oxidative stress and mitochondrial dysfunction have been suggested to underlie hyperglycemia-induced myocardial cell death by a number of studies (9, 12, 27, 51). However, the source of the oxidative stress, in addition to the exact nature of its relationship, if any, to mitochondrial-mediated cell death, remains unclear.

Recent findings from our group suggest that myocardium from diabetic patients has abnormal mitochondrial function, increased mitochondrial reactive oxygen species (ROS) production, and persistent oxidative/nitrosative stress (1). In the present study, we demonstrate that mitochondria in diabetic human myocardium have decreased mitochondrial Ca^{2+} tolerance and an increased propensity to Ca^{2+} -induced mPTP opening. In addition, activity of caspase-9, the mitochondrialmediated cell death protease, is elevated. These findings are the first to suggest that increased myocardial cell death described in diabetic patients may be mitochondrial-mediated and that

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mitochondrial-specific oxidative stress/redox imbalance may play a crucial role in regulating these processes.

METHODS

Clinical data and patient demographics. The University and Medical Center Institutional Review Board approved all aspects of patient recruitment and informed consent for this study. Cohorts evaluated were diabetic and nondiabetic patients at the East Carolina Heart Institute undergoing coronary bypass surgery (CABG) using extracorporeal circulation and hypothermic cardioplegic arrest. All demographic and clinical data are shown in Table 1. A patient was designated as having diabetes according to two major variables: 1) prior diagnosis of diabetes and 2) actively receiving treatment for diabetes. All preoperative diabetic medications are noted in Table 1. Patients with a history of prior myocardial infarct or structural heart disease, enlarged right atrium, history of arrhythmia, or left ventricular ejection fractions \leq 35% were excluded from this study.

Biopsy of right atrial appendage and tissue handling. After median sternotomy, and before institution of cardiopulmonary bypass, a purse-string suture was placed in the right atrial appendage to allow for placement of the venous cannula. A sample of the appendage directly superior to the purse-string was dissected and immediately rinsed in ice-cold saline to remove excess blood. Muscle tissue was then trimmed away from epicardium and pericardial fat, and a portion was placed in ice-cold *Buffer X* (see below) for mitochondrial measurements, and another was frozen in liquid N₂. This method ensures that all samples obtained for study are predominantly cardiac muscle and that they are rapidly processed (<90 s from time of dissection) to minimize protein and mRNA degradation.

Table 1.

	Nondiabetic Patients $(n = 12)$	Diabetic Patients $(n = 9)$
Demographics		
Age, yr	54.8 ± 2.4	59.1 ± 2.8
Sex, M/F	11/1	6/3
Race, C/AA/H	9/2/1	4/5/0
Clinical characteristics		
BMI	28.8 ± 1.5	33.5 ± 2.1
HbA1 _C	5.8 ± 0.1	$8.4 \pm 0.8*$
HDL	39.8 ± 3.4	37.0 ± 2.6
LDL	96.9 ± 10.3	97.4 ± 18.8
TG	155.1 ± 23.7	196.7 ± 30.4
LVEF, %	55.0 ± 1.5	55.6 ± 2.7
Hypertension, %	10 (83)	9 (100)
Preoperative medications, %		
Aspirin	9 (75)	7 (78)
β-Blocker	12 (100)	8 (89)
Clopidogrel	5 (42)	4 (44)
Other anticoagulant	0	1 (11)
Statin	8 (67)	8 (89)
Nitrates	3 (25)	3 (33)
Calcium channel blocker	0	1 (11)
Amiodarone	0	1 (11)
ACE inhibitor/ARB	4 (25)	6 (66)
Diuretic	1 (8)	3 (33)
Insulin	0	7 (78)*
DPP-4 inhibitor	0	1 (11)
Metformin	0	3 (33)*

Data shown in each column are means \pm SE; *n*, no. of patients. Nos. in parentheses are the percent of the total no. for each group for that variable. M, male; F, female; C/AA/H, Caucasian/African American/Hispanic; BMI, body mass index; HbA1_C, glycosylated hemoglobin; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TG, triglyceride; LVEF, left ventricular ejection fraction; ACE, angiotenisn-converting enzyme; ARB, angiotensin receptor blocker; DPP-4, dipeptidyl peptidase-4. **P* < 0.001.

Permeabilized fiber preparation. Portions of this technique have been described elsewhere (3) but have been adapted for application in human cardiac muscle and for specific measurements made in this study. After tissue harvest, muscle was placed in ice-cold *Buffer X* containing (in mM): 7.23 K₂EGTA, 2.77 CaK₂EGTA, 20 imidazole, 20 taurine, 5.7 ATP, 14.3 phosphocreatine, 6.56 MgCl₂·6H₂O, and 50 MES; pH 7.1. Muscle was then cut into strips ~4–6 mm long × 2–3 mm wide, placed in a solution of *Buffer X* containing 3 mg/ml collagenase type I, and incubated for 30–45 min at 4°C. Fiber bundles were then carefully trimmed of vascular and connective tissue, separated along their longitudinal axis, and permeabilized for 30 min in *Buffer X* + 50 µg/ml saponin (30 µg/ml saponin if a female patient) at 4°C. Fibers were then washed in ice-cold *Buffer Y* containing (in mM): 250 sucrose, 10 Tris·HCl, 20 Tris base, 10 KH₂PO₄, 2 MgCl₂·6H₂O, and 0.5 mg/ml BSA until time of measurement (<4 h).

Measurement of mitochondrial O_2 consumption, H_2O_2 emission, and Ca^{2+} uptake in permeabilized human myofibers. All mitochondrial measurements were performed at 37°C. The Oroboros O2K Oxygraph system (Oroboros Instruments, Innsbruck, Austria) was used for all mitochondrial O_2 (m O_2) consumption measurements. The mitochondrial H₂O₂ (mH₂O₂) emission and individual mitochondrial Ca^{2+} (mCa²⁺) uptake measurements were obtained using a spectrofluorometer (Horiba Jobin Yvon, Edison, NJ), equipped with a thermojacketed cuvette chamber. For tandem mO₂/mCa²⁺ measurements, we constructed a customized system by interfacing a spectrofluorometer (Photon Technology Instruments, Birmingham, NJ) with the O₂K by extending a fiber-optic light guide from the excitation monochromator into the respiration chamber of the O₂K. Another light guide was extended from the glass aperture of the respiration chamber directly into the emission detector of the spectrofluorometer. The excitation and emission light guides were oriented at 90° to minimize light scattering and for optimal signal acquisition.

The mO₂, mCa²⁺, and mH₂O₂ experiments in this study were all performed in Buffer Y with 100 µM ADP, 5 mM glucose, and 1 U/ml hexokinase present to keep the mitochondria in a permanent, submaximal phosphorylating state. Pyruvate, glutamate, and malate were provided as indicated in the legends for Figs. 1-4. For mH₂O₂ measurements, Buffer Y contained 10 µM Amplex Red (Invitrogen, Carlsbad, CA) and 1 U/ml horseradish peroxidase, and mH₂O₂ emission was calculated as outlined previously (3). For mCa^{2+} measurements, Buffer Y contained 1 µM Calcium Green 5-N (Invitrogen). At the start of the mCa²⁺ experiments, 1 µM thapsigargin was added to inhibit sarco(endo)plasmic reticulum Ca2+ -ATPase (SERCA), and 40 μ M EGTA added to chelate residual Ca²⁺ and to establish minimum fluorescence. Pulses of 4 nmol Ca^{2+} (CaCl₂) were sequentially added, and mCa²⁺ followed until mPTP opening as described previously (40) and as indicated in the legends for Figs. 1-4. At end of experiment, 1 mM CaCl₂ was added to saturate the probe and establish maximum fluorescence. Changes in free Ca2+ in cuvette during mCa²⁺ uptake were then calculated using the known K_d for Calcium Green 5-N and the equations established by Tsien (54) for calculating free ion concentrations using ion-sensitive fluorophores. At the conclusion of all experiments, fibers were rinsed in doubledistilled H₂O, lyophilized in a freeze-dryer (Labconco, Kansas City, MO) for >2 h, and weighed on a microscale. Data are expressed as picomoles per minute per milligram dry weight (mH₂O₂) or total nanomoles per milligram dry weight (mCa²⁺).

Immunoblot analysis of myocardial protein. Samples of frozen atrial appendage muscle were homogenized in $10 \times (wt/vol)$ buffer containing (in mM): 10 Tris base, 1 EDTA, 1 EGTA, and Complete Protease inhibitor cocktail (Roche, Mannheim, Germany). Protein was then separated using SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and subjected to immunoblot using Mito OxPhos antibody cocktail (Mitosciences, Eugene, OR), which recognizes discrete subunits of mitochondrial OxPhos complexes I (~20 kDa), II (~30 kDa), III (~39 kDa), IV (~45 kDa), and V (~50 kDa), in the electron transport system, and anti-glyceraldehyde-3-phosphate dehy-



Fig. 1. Use of permeabilized myofibers prepared from human atrial appendage biopsy as an experimental model of mitochondrial Ca2+ (mCa2+) uptake and retention capacity. A: representative experimental trace showing increases in Calcium Green 5-N fluorescence in response to 4-nmol pulses of exogenous Ca2+ in a cuvette containing permeabilized human heart fibers maintained in a submaximal phosphorylating state as outlined in METHODS in the presence of 1 µM thapsigargin [sarco(endo)plasmic reticulum Ca2+-ATPase inhibitor] and 1 μM Ru-360, an inhibitor of mCa²⁺ uptake. B: permeabilized heart fibers were incubated under identical conditions as in A, without Ru-360. Here, a tandem oximeter/spectrofluorimeter system was used to simultaneously measure O_2 consumption and Ca^{2+} uptake, and the traces shown were acquired simultaneously from the same preparation of permeabilized fibers. At the start of the experiment, a permeabilized fiber bundle (PmFB) + 100 µM ADP was added to the respiratory medium, followed by the substrates and Ca2+ where indicated. The trace on top shows fluorescence of Calcium Green 5-N in response to Ca²⁺ pulses, with a corresponding decrease in fluorescence after each pulse indicating mitochondrial uptake of Ca²⁺. The trace on bottom shows O₂ tension in respiratory medium (dark gray trace, y-axis on left) and the rate of O₂ consumption (light gray trace, y-axis on right) as each substrate and Ca2+ pulse (vertical lines) is added to the chamber. JO2, O2 flux/O2 consumption.

drogenase (Advanced Immunochemical, Long Beach, CA). Densitometry was performed using Image J software (48).

Assay of caspase-3,8,9 activity in myocardial homogenate. Samples of whole atrial muscle homogenate were prepared in identical buffer as for immunoblot and assayed for activity of caspases-3, -8, and -9 using commercial kit reagents and the procedure as outlined by the manufacturer (Biovision, Mountain View, CA).

Data analysis and statistics. Data shown in Table 1 and in Figs. 1–4 are expressed as means \pm SE. Categorical variables in Table 1 were compared using Fisher's exact test, and interval variables between both groups (diabetic and nondiabetic) were compared using the Student's unpaired *t*-test. Differences between both groups were considered statistically significant for P < 0.05.

RESULTS

Energetics of mCa^{2+} uptake using permeabilized human myofibers as a model. We first confirmed that uptake of exogenous Ca²⁺ in permeabilized myofibers prepared from human right atrial appendage is sensitive to the known mCa²⁺ uptake inhibitor Ru-360 and not thapsigargin, the inhibitor of Ca²⁺ uptake at sarcoendoplasmic reticulum (Fig. 1A). In addition, using a tandem oximeter/fluorimeter apparatus, we confirmed that mCa^{2+} uptake stimulates an increase in O_2 consumption when respiration is supported by pyruvate, glutamate, and malate in the presence of 100 µM ADP and an ADP-regenerative system to keep the mitochondria in a permanent, submaximal phosphorylating state (Fig. 1B). This concentration of ADP is similar to what has been demonstrated to be the approximate physiological concentration maintained in vivo (33). Note the rate of O_2 consumption (Fig. 1B, bottom) increases upon mitochondrial uptake of Ca2+ (compare red trace with only pyruvate, glutamate, and malate with red trace after Ca^{2+} bolus), but the rate of O₂ consumption plateaus and then remains constant upon further uptake of Ca^{2+} because the slight depolarization of mitochondrial $\Delta \psi$ brought about by Ca^{2+} uptake is matched by increased H⁺ extrusion from respiratory complexes supported by the increased NADH driven by Ca²⁺ activation of pyruvate dehydrogenase and α -ketoglutarate dehydrogenase (41). After a threshold concen-



Fig. 2. Mitochondria in cardiac tissue from diabetic patients have increased sensitivity to exogenous Ca^{2+} and increased H_2O_2 emission. Shown in *A* are representative experimental traces of mCa^{2+} uptake in permeabilized myofibers prepared from atrial appendage biopsies in nondiabetic (*top*) and diabetic (*bottom*) patients. *B* and *C*: the quantified amount of total mitochondrial Ca^{2+} retained before opening of mitochondrial permeability transition pore (mPTP) (*B*) and quantified rates of mitochondrial H_2O_2 emission in paired experiments (C). Data shown are means \pm SE, representative of n = 9-12, **P* < 0.05.

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tration of Ca^{2+} is reached in the mitochondrial matrix, the mPTP is triggered to open, causing the matrix to release the stored Ca^{2+} (Fig. 1*B*, *top*) as well as the labile pool of NADH generated from pyruvate and glutamate oxidation, thereby halting respiration (Fig. 1*B*, *bottom*).

Total mCa^{2+} retention capacity and mH_2O_2 in myocardial tissue from type 2 diabetic and nondiabetic patients. We then examined whether differences existed in mCa^{2+} retention capacity (i.e., Ca^{2+} sensitivity of mPTP) in myocardial tissue between diabetic and nondiabetic patients. An ~25% reduction (P < 0.05) in mCa^{2+} retention capacity was observed in diabetic myocardial tissue (Fig. 2, A and B). Under identical conditions, mH_2O_2 was ~2.5-fold greater (P < 0.05) in diabetic than in nondiabetic patients (Fig. 2*C*).

Mitochondrial respiratory enzyme content and mO_2 . Under identical experimental conditions as indicated in Fig. 2, rates of mO_2 were similar between diabetic and nondiabetic patients (Fig. 3A). The mitochondrial content also was unchanged between groups, as assessed by citrate synthase activity (Fig. 3B) and immunoblot for respiratory complexes in the electron transport system (Fig. 3, C and D).

Activities of caspase-3, -8, and -9. To establish whether caspase-dependent apoptotic pathways were elevated in diabetic myocardial tissue, we assessed the activities of caspases-8 and -9 and determined that diabetic patients had approximately twofold greater caspase-9 activity (P < 0.05) but similar caspase-8 activity compared with nondiabetic patients (Fig. 4). We then examined whether this increased caspase-9 activity in diabetic patients resulted in increased caspase-3 activity, the final "executioner" caspase, and determined that caspase-3 activity was not significantly greater in diabetic patients (P = 0.07).

DISCUSSION

The existence of a pathological state within the diabetic myocardium distinct from coronary artery disease has now been clearly established. It is commonly referred to as diabetic cardiomyopathy, a condition that, unless reversed, frequently leads to heart failure. Increased myocardial cell death is a prominent feature of all cardiomyopathies and is a major determinant in clinical outcomes (24). Given the well-known increase in levels of cell death in diabetic human hearts (14, 25, 37), we postulated that the source of this increased propensity for cell death may be stressed and/or vulnerable mitochondria. However, serious limitations to studying pathophysiology at the cellular level in human heart exist that make this assessment very difficult. Because biopsies of healthy human heart cannot be obtained, the issue of determining how best to



Fig. 3. Mitochondrial enzyme content and rates of O2 consumption under study conditions are unchanged in cardiac tissue from diabetic patients. A: quantified rates of submaximal state 3 O2 consumption (100 µM ADP) in permeabilized cardiac fibers energized with pyruvate, glutamate, and malate (PGM) and PGM + succinate (PGMS) from nondiabetic and diabetic patients. B: rates of citrate synthase activity in tissue homogenates prepared from both groups. C: representative immunoblot of cardiac tissue protein from 6 diabetic and 6 nondiabetic patients using a primary antibody cocktail that recognizes polypeptides from complex I-IV and the F₁F₀-ATPase in the electron transport system (green bands), with glyceraldehyde-3-phosphate dehydrogenase as loading control (red band). Protein-ladder standards are shown in lane 1 and lanes 14 and 15. D: densitometry analysis of OxPhos protein from each patient group. Quantified data shown are means \pm SE, representative of n = 8-10. AU, arbitrary units; OxPhos, oxidative phosphorylation.



Fig. 4. Activity of caspase-9 is increased in cardiac tissue from diabetic patients. Quantified rates of caspase-9 (*A*), caspase-8 (*B*), and caspase-3 (*C*) in cardiac tissue from nondiabetic and diabetic patients are given. Data shown are means \pm SE, representative of n = 9-12, *P < 0.05.

investigate the effect of a particular disease/condition in an extractive manner is challenging because there is no true "control group." In this study, all patients were having CABG due to severe coronary artery disease, taking numerous medications, and coming from a mixed clinical demographic, all of which could affect mitochondrial function, thereby confounding our study. After careful consideration, we determined the best strategy for investigating this question in a cross-sectional study design between nondiabetic and diabetic patients with coronary artery disease is to keep both groups similar with respect to medications, disease presentation, age, race, sex, etc., while isolating a singular clinical variable that is distinctly different between them. In this study, that single variable was hyperglycemia due to diabetes. With the use of this approach, it can be reasonably concluded that, if differences do exist in mitochondrial function, then the differences are probably due to the diabetes, and not one of the multitude of other clinical variables that exist in these patient cohorts. The findings of this translational study are the first to demonstrate 1) an increase in sensitivity of mPTP to Ca²⁺ in myocardial tissue of diabetic patients; 2) this increased mPTP sensitivity is associated with increased mitochondrial ROS production; and 3) the intrinsic caspase-9- but not extrinsic caspase-8-dependent cell death pathway is elevated in myocardial tissue of diabetic patients. These findings suggest that the increased cell death observed in diabetic myocardium is a result of distinct, mitochondrialmediated cell death pathways, possibly as a result of metabolic stress-imposed dysfunction in the mitochondria. Importantly, this dysfunction is present despite no difference in total mitochondrial content in cardiac tissue between diabetic and nondiabetic patients.

Recently, the importance of mitochondria in the cell's response to stress has been increasingly highlighted in a number of studies. Because cardiomyocytes contain so many mitochondria to meet their energetic requirements, this organelle is proving to be of monumental importance in the pathogenesis of diseases where metabolic stress and inflammation affect cardiac function and/or morphology (5, 6). Metabolic stressors such as hyperglycemia (12, 22, 44, 51) and dyslipidemia (8, 26, 34) have repeatedly been shown to cause increased mitochondrial-derived oxidative stress and cell death. Mechanisms by which these metabolic stressors cause alterations in the biochemistry of cardiac mitochondria remain a focus of intense research and are likely a collective result of a multifaceted degenerative process comprised of oxidative mitochondrial DNA damage (20), posttranslational modification of mitochondrial enzymes (10, 13), alterations in mitochondrial membrane architecture (55), and compromised antioxidant defenses (23, 27). Inflammatory cytokines such as tumor necrosis factor (TNF)- α , known to be elevated in patients with metabolic syndrome and type 2 diabetes, have also been shown to stimulate mitochondrial ROS (39, 53) and cardiomyocyte death (32, 42) in part through activation of a sphingolipid/ceramide-mediated lipotoxic pathway (53). However, the increased mitochondrial ROS and mPTP sensitivity to Ca²⁺ seen in diabetic heart tissue in the present study is unlikely to be driven by a ceramide-mediated pathway, since recent findings by Baranowski et al. (8) showed that ceramide levels are not increased in atrial appendage from obese, type 2 diabetic patients compared with obese, nondiabetic patients. It should be noted that those findings do not preclude the existence of other pathways by which TNF- α and inflammatory cytokines may be causing the increased mitochondrial ROS and mPTP sensitivity to Ca^{2+} .

From a mechanistic perspective, linking increased mitochondrial ROS/oxidative stress to increased Ca²⁺ sensitivity of mPTP in diabetic myocardium somewhat contradicts the paradigm in that increased mitochondrial ROS has been shown to be cardioprotective in some models, such as during ischemic preconditioning, by causing a decreased sensitivity of the mPTP to Ca^{2+} (7, 17). In this context, however, it is imperative that the temporal nature and magnitude of the increase in mitochondrial ROS/oxidative stress be considered. Transient increases in mitochondrial ROS likely mediate cardioprotection from ischemic preconditioning (7, 16), and low, subtoxic increases in mitochondrial ROS/oxidative stress for short periods of time (i.e., days) have been shown to be beneficial by eliciting a hormetic response and augmentation of cellular antioxidant defense capacity along with mitochondrial biogenesis (47). In diabetes, it is the sustained impact of metabolic and oxidative stress on mitochondria for prolonged periods of time (i.e., many months to years) that likely result in dramatic alterations of key mPTP components and its increased sensitivity to Ca²⁺. Indeed, critical residues of key components such as adenine nucleotide translocase (18, 29), cyclophilin D (38), and the mitochondrial-specific phospholipid cardiolipin (45, 46) all have been demonstrated to be altered by lipid peroxidation and/or oxidative stress. Hassouna and colleagues (31) recently showed that atrial appendage muscle from diabetic patients cannot be preconditioned by ischemia, and the authors attributed this to mitochondrial dysfunction and/or redox changes in the diabetic tissue. Glutathione depletion may also be playing a role in the relationship between mitochondrial ROS and Ca^{2+} sensitivity of mPTP (27). A recent study from our laboratory using similar cohorts of patients showed that cardiac glutathione levels are sharply decreased in diabetic patients (1), and this may be particularly stressful to mitochondria, which have to rely on production and transport of glutathione from cytosolic stores to maintain redox balance. Nevertheless, a detailed understanding of the relationship between mitochondrial ROS and Ca^{2+} sensitivity of mPTP is complex and incomplete, and further investigation is needed.

It is very plausible that changes in expression and/or biochemistry of key mPTP protein components are altered in the diabetic human heart. Williamson et al. (56) recently demonstrated that cyclophilin D expression is increased in the diabetic rat heart (56). Interestingly, another recent study showed that cyclophilin D protein was more closely bound to the inner membrane of volume-overloaded rat hearts and that treatment of cardiac mitochondria with tert-butyl hydroperoxide, a global oxidant, caused increased binding of cyclophilin D to the inner membrane in a dose-dependent manner (40). However, because many of the molecular underpinnings of the increased Ca2+ sensitivity of mPTP in diabetic heart are likely due to the posttranslational modifications of mPTP components and/or redox-related effects discussed above, it is worth emphasizing that the large number of previous studies that have only explored changes in expression and/or content of these components in diabetic hearts (e.g., using immunoblot) are of indeterminate value because they don't take these modifications into account.

Unlike the clearly defined, singular role of mPTP in necrosis, mitochondria coordinate apoptosis using a variety of pathways, all of which can differ according to the type and duration of the stress imposed. A unifying feature of most of the mitochondrial-mediated apoptotic programs is that they typically occur through a mitochondrial outer membrane permeabilization step, which releases intermediary proteins (e.g., cytochrome c, AIF) from the inner membrane space into the cytosol, thereby complexing with Apaf-1 and caspase-9 to form a larger complex that then, in turn, activates effector caspases-3 and -7 to ultimately cause cell death. Cytokines and other ligand/receptor-mediated reactions on the plasma membrane initiate the extrinsic pathway, culminating in the activation of caspase-8, which then can activate caspase-3 downstream (15, 35, 36). In the present study, activity of caspase-9, but not -8, was elevated in myocardial tissue from type 2 diabetic patients. This finding would suggest that it is largely the mitochondrial-mediated apoptotic program that underlies the increased apoptosis observed in diabetic human heart. However, there are numerous factors upstream of caspase-8 and -9 that were not determined in this study due to limitations in the quantity of myocardial tissue available, so it is difficult to fully appraise whether the mitochondrial-mediated pathway is the sole determinant of cardiomyocyte apoptosis in diabetic patients, in addition to which of the intermediary proteins are involved at the start of the pathway.

Finally, it should be mentioned that, because this study was performed using permeabilized fibers from intact tissue, the findings are reflective of mitochondria in the tissue as a whole. As such, the well-documented differences in structure, morphology, and function that exist between subsarcolemmal and interfibrillar populations of mitochondria were not taken into account (49), and this may be of importance because of the increasing evidence suggesting that these discrete populations are differentially affected in heart failure (50) and diabetes (19, 56). Nevertheless, because the findings of the present study are representative of mitochondria in the entire tissue as it exists in its architectural and biochemical state in vivo, it is still an important first step in understanding the role of mitochondria in mediating cell death in the diabetic human heart. In addition, these findings provide evidence that mitochondria are a compelling target for treatment of cardiomyopathy in diabetic patients and that therapeutic compounds such as mitochondrial-targeted antioxidants (2, 49, 57) and others in the new generation of "mitochondrial medicine" may be uniquely suited for use in diabetic patients.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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