Chronically elevated glucose compromises myocardial mitochondrial DNA integrity by alteration of mitochondrial topoisomerase function

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Medikayala S, Piteo B, Zhao X, Edwards JG. Chronically elevated glucose compromises myocardial mitochondrial DNA integrity by alteration of mitochondrial topoisomerase function. Am J Physiol Cell Physiol 300: C338–C348, 2011. First published December 1, 2010; doi:10.1152/ajpcell.00248.2010.—Mitochondrial dysfunction has a significant role in the development and complications of diabetic cardiomyopathy. Mitochondrial dysfunction and mitochondrial DNA (mtDNA) mutations are also associated with different types of cancer and neurodegenerative diseases. The goal of this study was to determine if chronically elevated glucose increase in mtDNA damage contributed to mitochondrial dysfunction and identify the underlying basis for mtDNA damage. H9c2 myotubes (a cardiomyoblast-derived cell line) were studied in the presence of 5.5, 16.5, or 33.0 mM glucose for up to 13 days. Tests of mitochondrial function (Complex I and IV activity and ATP generation) were all significantly depressed by elevated media glucose. Intramitochondrial superoxide and intracellular superoxide levels were transiently increased during the experimental period. Annexin V binding (a marker of apoptosis) was significantly increased after 7 and 13 days of high glucose. Thirteen days of elevated glucose significantly increased mtDNA damage globally and across the region encoding for the three subunits of cytochrome oxidase. Using mitochondria isolated from cells chronically exposed to elevated glucose, we observed significant increases in topoisomerase-linked DNA cleavage. Mitochondria-dependent DNA cleavage was significantly exacerbated by H2O2 and that immunoprecipitation of mitochondrial extracts with a mtTOP1 antibody significantly decreased DNA cleavage, indicating that at least part of this activity could be attributed to mtTOP1. We conclude that even mild increases in glucose presentation compromised mitochondrial function as a result of a decline in mtDNA integrity. Separate from a direct impact of oxidative stress on mtDNA, ROS-induced alteration of mitochondrial topoisomerase activity exacerbated and propagated increases in mtDNA damage. These findings are significant in that the activation/inhibition state of the mitochondrial topoisomerases will have important consequences for mitochondrial DNA integrity and the well being of the myocardium.

diabetes; mitochondria; mitochondrial DNA damage; topoisomerase; DNA damage; hyperglycemia

DIABETIC CARDIOMYOPATHY (DCM) is characterized by the development of a myopathy initially manifested as diastolic dysfunction, but evolving into increased cavity dilation and mural thinning that is reflective of decompensated eccentric hypertrophy. DCM is associated with abnormal cardiac function, elevated apoptosis, and loss of cardiac mass in both human and animal models of diabetes (19, 21, 22). Mitochondrial dysfunction has a significant role in the development and complications of DCM (22, 46, 51). Mitochondrial dysfunction and mitochondrial DNA (mtDNA) mutations are also associated with other diseases, including Leigh-like syndrome, several types of cancer, and neurodegenerative diseases such as amyotrophic lateral sclerosis and Leber’s hereditary optic neuropathy (37, 39, 71). It has been reported that the mtDNA mutation rate is higher in diabetic patients than healthy individuals (50). Several studies have found that diabetes elevates oxidative stress, which is thought to contribute to mitochondrial dysfunction (49, 56). Less clear are the mechanisms leading to failure. Although the mitochondria have developed defense mechanisms to manage ROS and repair mtDNA, it is possible that chronic hyperglycemia may overwhelm their ability to manage the damage (24). This suggests that there may be limits to adaptability or that the chronic hyperglycemia ultimately produces an accumulation of errors from which the mitochondria are unable to cope.

In postmitotic cells, the mitochondrial genome continues to replicate about once a month (12). Mitochondrial DNA is thought to be at greater risk for ROS-induced damage due to its close proximity to the electron transport chain. Unlike genomic DNA, mammalian mtDNA does not contain introns that may serve to absorb damage from chronic oxidative stress. Indeed the “mitochondrial theory of aging” is centered on the idea of the accumulation of mtDNA mutations with a concomitant increase in mitochondrial oxidative stress, creating a “vicious cycle” that accelerates this process. Separate from this, investigations have used transgenic mice expressing “proofreading deficient” forms of mitochondrial DNA polymerase (mtDNA-Polβ). In those studies, cardiac cells accumulated mtDNA mutations at an accelerated rate without a change in oxidative stress, but with significant increases in apoptosis and heart failure (73). In a separate study, overexpression of 8-oxoguanine glycosylase (a DNA repair enzyme) was protective against Ang II-induced mtDNA damage and apoptosis (55). Although not diabetic, these models suggest that any increase in mtDNA mutations may initiate apoptosis.

Poor glycemic control is a negative prognosticator in diabetic patients. Hyperglycemia is a consistent feature of several animal models of diabetes including db/db mice, fatty Zucker, OLETF, and the Goto-Kakizaki rats. Although in db/db mice and fatty Zucker rats, myocardial glucose usage is decreased in favor of increased fatty acid oxidation, increased glucose flux through the pentose phosphate pathway has been reported (1, 10, 43, 47, 58, 67). Hyperglycemia-induced mitochondrial dysfunction has been suggested as one contributing factor to accelerated myocardial apoptosis (9, 53, 56). To date, cultured cell studies have examined the impact of elevated glucose for only short periods, and even fewer have examined the impact of diabetes on mtDNA. The etiology for the accumulation of mtDNA mutations and deletions remains obscure, but the impact on mitochondria biogenesis and function is substantial (13, 28, 66). To determine if elevated glucose could alter
mitochondrial function and mtDNA integrity, cultured H9c2 myotubes were studied in the presence of elevated glucose for up to 13 days. We determined that chronically elevated glucose increased mtDNA damage by alteration of mitochondrial topoisomerase function.

**MATERIALS AND METHODS**

A detailed description of the protocols and methods used is provided in an online supplement.

**Cell culture.** These experiments used H9c2 myotubes maintained in culture in 5.5, 16.5, or 33.0 mM glucose for 1, 3, 7, or 13 days; osmolality was balanced by the addition of mannitol. To permit comparison across time points, all cells were plated at one time; additions were made as illustrated in Fig. 1. Where indicated, 5.5 mM/hyp is LG-DMEM plus mannitol to raise the osmotic pressure to be equivalent to the 33 mM/iso is isosmotic LG-DMEM alone; 5.5 mM/hyp is LG-DMEM additions were made as illustrated in Fig. 1. Where indicated, 5.5 mM glucose. Where no designation is made in the results, the control group is 5.5 mM/hyp.

**Mitochondrial function.** ATP production was measured by the CellTiter-Glo luminescent assay (Promega, Madison, WI). Values presented are means ± SE of normalized to controls of the arbitrary optical density or fluorescence units. Cellular NADH levels were determined by UV-autofluorescence (λex/λem; 360/450 nM; Ref. 52). Succinate dehydrogenase was determined by a stop time assay, using thiazolyl blue tetrazolium; absorbance was recorded at 560 nm, with 690 nm used as a reference (15). Mitochondrial Complex I activity was determined following NADH oxidation at 340 nm; Complex II activity was determined by measuring cytochrome c reduction at 550 nm using succinate as a substrate (4). Cytochrome oxidase (Complex IV) was measured by the oxidation of reduced cytochrome c as we previously described (54).

**Apoptosis.** Three measures of apoptosis were made: 1) by flow cytometry of AnnexinV binding, 2) by DNA fragmentation, 3) by EvaGreen that is a cell-impermeant dye that fluoresces on binding to DNA of late stage apoptotic and necrotic cells.

**Superoxide determinations.** MitoSox (2.5 μM; Invitrogen) was used to determine mitochondrial superoxide production. Intracellular O₂⁻ production was determined using 20 μM dihydroethidium (DHE). The development of fluorescence was measured using a Tecan M200 plate reader (MitoSox: λex/λem; 510/580; DHE: λex/λem; 480/573).

**mtDNA damage.** Damage across the length of the mtDNA was assessed by long-range PCR (LRPCR), as described previously (18). In brief, the rationale is that any lesion (strand breaks, base modifications, and apurinic sites) will stop a thermostable DNA polymerase capable of generating a long DNA product. The copy number derived from this amplification was compared with the copy number of a short-range PCR (SRPCR) product (150–250 bp) that is unlikely to contain any lesions. By this approach, an increasing amount of mtDNA damage is inversely related to DNA yield as shown in Fig. 5A. The SRPCR primers amplified the mtDNA in the 12S rRNA coding region. The primers used are listed in Table 1 in the online supplement. mtDNA damage and mitochondrial copy number were derived by the 2ΔCt method, from the comparison of LRPCR: SRPCR and SRPCR:β-actin, respectively. mtDNA yield and mitochondrial copy number are expressed as means ± SE and normalized to 5.5 mM glucose/isosmotic control group.

**DNA cleavage assay.** DNA cleavage was determined by two protocols. The first used a conventional approach using linar DNA as described by Kao et al. (30). The second approach used the LRPCR protocol as described above. For the H₂O₂ and topoisomerase inhibitor experiments, isolated mitochondria were preincubated for 60 min at 37°C with those reagents or vehicle before the addition of DNA and incubated for an additional 30 min at 37°C. The reaction products were electrophoresed on an agarose gel.

**Statistical analysis.** Statistical analyses were performed using NCSS Software (NCSS, Kaysville, UT). Where appropriate, Student’s t-test or ANOVA was used; post hoc analysis was done using a Fisher’s least significant difference analysis. Values presented are means ± SE, and statistical significance was set at P < 0.05.

**RESULTS**

Glucose-induced alterations in mitochondrial function and oxidative stress.

These experiments used H9c2 myotubes maintained in culture in 5.5, 16.5, or 33.0 mM glucose for up to 13 days. At harvest, increased glucose levels did not significantly alter protein levels in the H9c2 cells (data not shown). Mitochondrial dysfunction was evidenced by significant decreases in cellular ATP production (Fig. 2A) and decreased succinate dehydrogenase activity (Fig. 2B). In conjunction with this, we observed that chronically elevated glucose significantly depressed mitochondrial Complex I activity but not Complex II activity (Fig. 2C). Increased media glucose also resulted in a rise in cellular NADH levels after 13 days in the 33 mM group (Fig. 2D), suggesting reduced electron flow through the electron transport chain. All of these results are indicative of mitochondrial dysfunction.

Glucose-induced increases in cellular and mitochondrial superoxide levels have been reported over the short term (9). MitoSox and DHE are cell-permeant dyes that partition to the mitochondria and cytosol, respectively, and fluoresce in the presence of superoxide. We observed that MitoSox fluore-
cence (mitochondrial superoxide) was transiently increased in response to chronically elevated glucose (Fig. 3, A and B). Interestingly, the apogee of the response to 16.5 mM glucose lagged behind the response to 33 mM glucose (Fig. 3A). Similarly, transient increases in DHE fluorescence (intracellular superoxide; Fig. 3C) as well as extracellular H$_2$O$_2$ levels (Fig. 3D) were observed.

Oxidative stress is the imbalance between reactive oxygen species (ROS) generation and intracellular buffers, and increased oxidative stress is thought to promote apoptosis. We did not observe the apoptotic indicative “laddered DNA” pattern using total DNA from high glucose-treated cells (Fig. 4A).

EvaGreen, a cell impermeant dye that binds to nucleic acids of damaged cells was not altered by chronically elevated glucose.
Fig. 4. Chronically elevated media glucose induces an increase in AnnexinV binding. 
A: DNA fragmentation analysis of genomic DNA from H9c2 myotubes maintained under varying glucose concentrations for 13 days. 
B: EvaGreen binding to double-strand DNA in cells exposed to elevated glucose for 1, 3, 7, or 13 days. Cell impermeant EvaGreen was added and fluorescence detected in the 96 well plates as described in MATERIALS AND METHODS. Values presented are means ± SE of fluorescence normalized to 5.5 mM/1 day controls. C: flow cytometric detection of AnnexinV binding. Values presented of 7–24 samples and are means ± SE of % gated cells. D: caspase 3/7 activity in cells exposed to elevated glucose for 1, 3, 7, or 13 days. *P < 0.05 compared with respective 1 day control.

Fig. 5. Elevated media glucose increases mitochondrial mtDNA damage. 
A: long-range QPCR analysis to detect mitochondria DNA (mtDNA) damage across the length of the mitochondrial genome. mtDNA damage is inversely related to DNA yield. B: mitochondrial copy number. Values are means ± SE of 12 samples and normalized to the 5.5 mM isosmotic glucose controls. C: representative long-range PCR (LRPCR) mtDNA reaction products.

(Eq. 4B), also indicating that there was not a large scale cell death of H9c2 cells. In contrast, using flow cytometry, we did observe significant increases in AnnexinV binding after 7 and 13 days of elevated media glucose (Fig. 4C), as well as a transient increase in caspase 3/7 activity (Fig. 4D).

Elevated media glucose caused mitochondrial mtDNA damage. Nomiyama et al. reported that the mtDNA mutation rate is higher in diabetics than healthy individuals and we also observed increased myocardial mtDNA errors in GK diabetic rats (25, 50). For both glucose concentrations used, chronically elevated glucose significantly decreased mtDNA yield, which is reflective of increased mtDNA damage (Fig. 5A). The 33 mM glucose group but not the 16.5 mM glucose group also had a significant increase in mitochondrial copy number (Fig. 5B). Shown in Fig. 5C is a representative gel of the amplified mtDNA 15-kb products. Although some minor bands were observed, more so with the 33 mM glucose group, no consistently reproduced deletion bands were observed. This is important since it suggests that no regional “hot spots” or areas of vulnerability were consistently generated by elevated glucose. Using primers that spanned the mtDNA region encoding the three subunits of the cytochrome oxidase as well as ATPase subunit 6, we observed that chronically elevated glucose also significantly increased mtDNA damage within this region (Fig. 6A). Associated with this, complex IV (cytochrome oxidase) activity was significantly depressed by chronically
elevated glucose (Fig. 6B). Shown in Fig. 6C is a representative gel of the amplified mtDNA 4.6-kb products.

Topoisomerases mediated mtDNA damage. Although it has been accepted that ROS directly attacks mtDNA damage as the singular cause of mtDNA damage, to date the mechanism of mtDNA damage has not been established. To examine this, we used two in vitro assays to determine DNA cleavage. Mitochondria were isolated from H9c2 myotubes and maintained in 5.5, 16.5, or 33 mM glucose for 13 days. The mitochondrial isolation protocol was free of nuclei, which is a source of topoisomerase enzyme activity (Fig. 7B). In the conventional DNA cleavage assay, isolated mitochondria were incubated with linear DNA and the band intensity was determined following electrophoresis. Mitochondria isolated from H9c2 cells chronically exposed to elevated glucose media produced significantly more mitochondria-dependent DNA cleavage (Fig. 7A). This was found to be an enzymatic reaction as preincubation with proteinase K abrogated mitochondria-dependent DNA cleavage (see Fig. S1A in the online supplement). By a second approach, plasmid DNA was incubated with isolated mitochondria and then an aliquot of the reaction mixture was used in a modification of our LRPCR protocol. In mitochondria chronically exposed to elevated glucose, we observed a significant decrease in DNA yield reflective of an increased amount of DNA damage (Fig. 7A, “PCR”). Acute exposure of isolated mitochondria to increased glucose or mannitol did not significantly alter mitochondria-dependent DNA cleavage (see Fig. S1B in the online supplement).

Three unique topoisomerases and endonuclease G have been localized to the mitochondria and all can cleave DNA. To begin to examine their potential contributions DNA was incubated with isolated mitochondria using an in vitro assay. Significant mitochondria-directed DNA cleavage was observed, with greater degradation in the absence of Mg\(^2+\) (Fig. 8A). Endonuclease G requires Mg\(^2+\) to be active, and these findings indicated that it did not contribute to DNA cleavage, whereas topoisomerases may be responsible for DNA cleavage within the mitochondria (61, 70). It has been reported that ROS may
inhibit the topoisomerase religation step once the enzyme-DNA complex has been formed (40). If true, then ROS should enhance the DNA cleavage function of the topoisomerases. To test this, isolated mitochondria were incubated in the absence or presence of H2O2 for 60 min prior to the addition of linear DNA. We observed that under these conditions, 50% H9262 MH2O2 enhanced mitochondria-dependent DNA cleavage, but did not alter DNA band intensity in the absence of mitochondria (Fig. 8B). In contrast, acute preincubation of mitochondria with 100 mM glucose or mannitol did not alter mitochondria-dependent DNA cleavage, indicating that glucose induced alterations in cellular metabolism and not just the presence of glucose influenced mitochondrial topoisomerase function (see Fig. S1B in the online supplement).

Three unique topoisomerases have been indentified within the mitochondria. Immuno precipitation of isolated mitochondria with an antibody specific to TOP1 significantly decreased mitochondria-dependent DNA cleavage, indicating that at least a portion of the mtDNA degradation could be attributed to TOP1mt (Fig. 8C).

Sobuzoxane is a topoisomerase II inhibitor that binds to the topoisomerase-DNA complex and prevents the religation step with the consequence of increasing DNA strand breaks (2, 34). When incubated in the presence of sobuzoxane, significantly more mitochondria-dependent DNA cleavage was observed compared with extract alone, suggesting that topoisomerase II also contributed to mitochondria-dependent DNA cleavage (Fig. 8D). Similar results were obtained using other topoisomerase II inhibitors, including 10 μM Maleimide (data not shown) or Novobiocin (Fig. 9A). Chronic exposure to Novobiocin was deleterious. H9c2 cells exposed to 33 mM glucose for 13 days compromised ATP production, and this effect was further exacerbated by the presence of different concentrations of Novobiocin (Fig. 9B).

Fig. 8. In vitro assays for mitochondrial-directed DNA cleavage. A: DNA cleavage is not Mg2+-dependent. Isolated mitochondria were incubated in the presence (3 mM MgCl2) or absence of Mg2+ (0.5 mM EDTA). Values presented are means ± SE and normalized to the no mitochondria/+ Mg2+ group. *P < 0.05 compared with respective no mitochondria control; #P < .0.5 compared with + mitochondria/+Mg2+. B: H2O2 increases mitochondrial-dependent DNA damage. Mitochondria were preincubated in the absence or presence of 50 μM H2O2 prior to the addition of DNA. *P < 0.05 compared with respective no mitochondria/H2O control. #P < 0.05 compared with + mitochondria/+H2O. C: TOP1mt participates in mitochondrial dependent DNA cleavage. Isolated mitochondrial extracts were incubated with anti-TOP1 before the addition of Protein-G to extract the antibody bound material. *P < 0.05 compared with no extract; #P < 0.05 compared with +Mito alone. D: sobuzoxane increased mitochondrial-dependent DNA damage. Mitochondria were preincubated in the absence or presence of sobuzoxane (10 nM or 1 μM) before the addition of DNA. Values presented are means ± SE of 5–14 samples and normalized to no mitochondria control. *P < 0.05 compared with no extract control, &P < 0.05 compared with +Mito group.

Fig. 9. A: novobiocin increased mitochondrial-dependent DNA damage. Mitochondria were preincubated in the absence or presence of novobiocin (10 or 100 μM) before the addition of DNA. Values presented are means ± SE and normalized to no mitochondria control. *P < 0.05 compared with no extract control. #P < 0.05 compared with +Mito group. B: cellular ATP production in H9c2 cells exposed to 5.5 (open bars) or 33 mM (shaded bars) glucose for 13 days, in the presence of increasing doses of novobiocin. Values are means ± SE of 8 samples and normalized to the 5.5 mM glucose controls. *P < 0.05 compared with respective 5.5 mM glucose control. #P < 0.05 compared with 33 mM glucose/no Novobiocin group.
Mitochondrial dysfunction has a significant role in the development and complications of DCM (22, 46, 51). The mtDNA mutation rate has been reported to be higher in diabetic patients than healthy individuals and in ventricular mtDNA from the diabetic GK rats (25, 50). Less clear are the mechanisms that lead to failure since the heart possesses several distinct mechanisms for managing oxidative stress and DNA damage. The major findings of the present study are that even mild increases in glucose presentation will compromise mitochondrial function as a result of a decline in mtDNA integrity. Separate from a direct impact of oxidative stress on mtDNA, ROS-induced alteration of topoisomerase activity appears to propagate mtDNA damage.

Myocardial glucose usage is altered by diabetes and chronic hyperglycemia is common to several animal models of diabetes, including fatty Zucker, OLEFT, and Goto-Kakizaki rats as well as db/db and ob/ob mice (1, 10, 43, 47, 67). Mechanistic studies in cultured cells have examined the impact of elevated glucose for only short time periods (9, 53, 56). Although it does not replicate the chronic state that diabetes is, we extended the experimental time frame in an attempt to better understand how the cell manages chronically elevated glucose. Within this context, the cell populations appeared to remain relatively stable. However, cellular ATP production was compromised as an early event at both elevated concentrations of glucose studied. Others have observed transient hyperglycemia-induced decreases in ATP production in neuronal cells (56). Hyperglycemia is not alone in compromising mitochondrial function, as Rachek et al. (53) reported that elevated palmitate significantly decrease ATP levels in L6 muscle cells.

It has been maintained that elevated ROS is the underlying mechanism for mtDNA damage as a result of increased glucose-derived electron flux through the electron transport chain (ETC). More recently, Block et al. (6) reported that Nox4 is localized to the mitochondrial intermembrane, and in cultured mesangial cells exposure to high glucose increased mitochondrial Nox4 expression. This has important consequences: NAD(P)H is readily accessible to the intermembrane region, and, unlike other Nox isoforms, Nox 4 has constitutive activity that is not dependent on activation by the p47phox or p67phox subunits (45). Although glycolysis declines in the diabetic heart, glucose flux though the pentose shunt pathway is elevated in the diabetic heart to increase NAD(P)H production (58). In combination, this may represent a significant source of ROS localized to the mitochondria. In the present study, we observed that increases in detectable ROS were transient and this was reflected in both whole cell (DHE) and mitochondrial (MitoSox) compartments. Similar glucose-induced transient increases in ROS have been observed by others, in dorsal root neurons (56) or H9c2 cells (9). These differences may be a function the different GLUT receptor isoforms at the cell surface, intracellular buffering capacity for ROS, and their response to chronically elevated media glucose. In a cytochrome c-deficient cell line, MitoSox fluorescence was decreased throughout the 13-day time frame (data not shown). It is interesting that the significant rise in superoxide in cells exposed to 16.5 mM glucose lagged compared with the response to 33 mM glucose, suggesting a lesser stress took longer to develop a significant response.

Another consideration is that MitoSox partitions to the mitochondria because it is hyperpolarized with respect to the cytoplasm, in a manner similar to the fluorescent dye JC1. As mitochondria transition on the path to degradation they depolarize, which is the underlying basis for using JC1. It may be that the MitoSox dye does not move into the mitochondria at the same rate once the mitochondria begin to depolarize. Also, we have not at all examined the role of autophagy. It may be that if this is significantly increased, the MitoSox signal may be quenched as the mitochondria enter the autophagosome.

It has been reported that increased glucose will initiate an early wave of apoptosis (9, 20, 33). By two measures of apoptosis, DNA fragmentation and EvaGreen staining, we did not observe evidence for apoptosis, a finding both consistent with (57) and in contrast to (9, 53) what others have reported. However, using flow cytometry and measuring AnnexinV binding, glucose-induced apoptosis was evident. The first two measures are indicative of late stage of apoptosis, whereas increased AnnexinV binding represents an earlier event in the apoptotic cascade. Also, flow cytometry is likely to be a much more sensitive approach than DNA fragmentation. Our findings that a significant increase in AnnexinV binding occurred beginning at day 7 suggests that the onset of large scale apoptosis may have been beyond the time frame of the experimental protocol. Alternatively, these findings may suggest a wave of apoptosis very shortly after exposure to high glucose that is followed by buffering of the insult or possibly by activation of autophagy. This latter interpretation would more closely match our caspase activity pattern and the findings of Cai et al. (9) who, using image analysis, observed an early wave of apoptosis induced by elevated glucose. Similarly, Fiordalsio et al. (20) reported elevated glucose will activate p53 and induce apoptosis within a short time frame. It is interesting to note that for both studies, the apogee of the response to the lower concentration lagged behind the higher concentration, suggesting a time-stress dependency on the cellular response.

Early observations that mitochondria were unable to repair UV-induced damage of mtDNA suggested a lack of a DNA repair function within mitochondria (11, 36). Although mitochondria are poor at repairing this one type of lesion, they are capable of other forms of DNA damage repair (17). Thus the significant increase in mtDNA damage we observed indicated that the rate of mtDNA damage overwhelmed the functional mtDNA repair capability.

Increased glucose promoted degradation of mitochondrial function in part by an increase in mtDNA mutations. This was observed across the whole of the mtDNA genome as well as the region encoding cytochrome oxidase, and this corresponded to depressed cytochrome oxidase activity. Similarly, Complex I, which contains seven mitochondrial encoded proteins, had significantly depressed activity following chronic elevated glucose. In contrast, Complex II, whose proteins are encoded by the nucleus, was not significantly affected. The presence of mtDNA damage may lead to altered stoichiometry of the cytochrome oxidase complex and compromise its function. Decreased cytochrome oxidase subunit I protein levels have been reported in a model of streptozotocin-induced diabetes (29). Although it is possible that expression of mitochondrial proteins was decreased by elevated glucose, this aspect was not examined within this study. Another consideration was
the possible presence of point mutations. We previously observed point mutations within the coding sequence of Complex IV subunit 3 in the left ventricle of the diabetic GK rat that were associated with amino acid substitutions or the formation of truncated proteins (25). This type of alteration may not only alter the protein-protein interaction within the mature cytochrome oxidase, but also possibly interfered with its initial formation within the ETC, which requires the coordinated effort of several chaperone-like proteins.

It might be expected that ROS-induced damage of mtDNA would be a random event. However, Gokey et al. (23) reported that mtDNA deletions in aged skeletal muscle mtDNA occurred with a greater frequency in regions downstream from the origins (heavy and light) of replication. Since replication requires mtDNA to be “unwound” and not protected by histone-like proteins such as Tfam, Gokey’s data suggest that these regions may be more vulnerable. Others have noted an accumulation of mtDNA deletions centered on regions containing direct repeats (26). Although the mechanisms producing deletions may be different from the formation of point mutations or DNA strand breaks, these data suggest that there may regional vulnerabilities for the formation of mtDNA mutations. Our LRPCR and MRPCR protocols observed that some smaller bands were amplified in some samples but no consistent pattern was identified. At this point we have not subjected the smaller bands to sequence analysis to determine if those fragments originated from the same region.

Despite the presence of mtDNA damage at 16.5 mM glucose, no changes in mitochondrial copy number were observed within the time frame of these experiments. In concert with this, we did not see a rise in apoptotic markers comparable to that observed at the higher (33 mM) glucose dose. However, in the 33 mM group, a significant increase in mitochondrial copy number was observed. Mitochondria copy number has been used as one estimate of the number of mitochondria present within the cell, albeit with many limitations. The mitochondria are responsive to many different influences. Our observations may reflect an attempt by the cell to recover from its dysfunction (increased mtDNA damage) as well as promotion of mitochondrial biogenesis by other participating factors. We observed a significant decline in ATP production as an early mitochondrial biogenesis (27).

Chronic hyperglycemia may not be well tolerated over the longer term. In part, this may be due to exhaustion of the endogenous buffering systems. Alternatively, it may be that a transient signal is prolonged by activation of a pathway to exacerbate the initial insult. These findings are important since heretofore it has been thought that mtDNA damage was only the result of a direct attack of ROS on mtDNA. Our finding of only transient increases in mitochondrial ROS levels suggests activation of alternative processes. With the use of an in vitro protocol, mitochondria isolated from cells exposed to elevated glucose produced greater DNA cleavage compared with controls. That these processes were not Mg²⁺−dependent minimizes the role endonuclease G may have, while pointing toward a significant role for the mitochondrial topoisomerases in mediating glucose-induced mtDNA damage.

Using a TOP1 antibody, we found that immunoprecipitation of TOP1mt decreased mitochondria-dependent DNA cleavage. Topoisomerases are highly conserved proteins present in all organisms. Topoisomerases resolve the topological difficulties of DNA replication by allowing the double helix to pass through itself. This entails a complex reaction order where 1) DNA is bound, 2) DNA strand breaks are induced, 3) the DNA structure is rotated to relieve torsion-induced stress, and 4) religation of DNA strands. In postmitotic cells, the mitochondrial genome continues to replicate about once a month, and resolving the topology of mtDNA replication would be almost insurmountable in the absence of the topoisomerases (12). Three unique isoforms of topoisomerase have been demonstrated in the mitochondria: topoisomerase Iβ (a type IIA), TOP1mt (a type IB), and TOP3α (a type IA) (44, 68, 74). Although topoisomerase activity is essential to the cell and generally protective, it may also be genotoxic (16). Topoisomerase inhibitors or poisons act via a variety of mechanisms to alter topoisomerase function. Some, such as doxorubicin, permit DNA binding and cleavage but not the religation or release of the relaxed DNA structure. This has the consequence of inducing DNA strand breaks (DSB) without a comparable increase in DNA repair. Consistent with this concept, we observed that sobuzoxane and novobiocin (topoisomerase II inhibitors) exacerbated mitochondria-dependent DNA cleavage and indicate that the activation/inhibition state of mitochondrial topoisomerases is critical to the functional wellbeing of the diabetic myocardium.

We observed significantly increased DNA cleavage in isolated mitochondrial extracts from chronically elevated glucose-treated cells compared with controls. These results indicate that chronically elevated glucose brought about the activation of topoisomerase DNA cleavage activity. A second in vitro test, using circular DNA and an LRPCR approach, yielded similar results: a lower yield of DNA reflective of increase mtDNA damage. In contrast, acute incubation of naive mitochondrial extracts with elevated glucose or mannitol was without effect, indicating that this was not a direct effect (see Fig. S1B in the online supplement). It has been reported that oxidative stress alters nuclear topoisomerase activity to stabilize the topoisomerase/DNA complex while inhibiting the religation process (40). Consistent with this, H₂O₂ treatment exacerbated mitochondria-dependent DNA cleavage (Fig. 8B). Our findings of...
transient increases in ROS could serve to activate topoisomerases to propagate mtDNA strand breaks beyond the time frame of elevated mitochondrial ROS. It has also been shown that single-strand DNA nicks act as a topoisomerase poison to localize topoisomerases and to induce cleavage on the opposite strand (16, 31). This would not only enhance DNA cleavage by topoisomerases in the mitochondria but would also hold mtDNA “in an open conformation” longer and expose it to further ROS attack. Thus any ROS-induced damage may serve to localize and activate mitochondrial topoisomerases to the damaged mtDNA and further exacerbate mtDNA damage beyond the time frame of the insult.

Our findings, using the LRPCR approach, found that the Pfu DNA polymerase was unable to amplify across DNA breaks and this has some important consequences. Both mammalian DNA and RNA polymerases are unable to read across DNA breaks (62, 64). As well, polymerases are unable to progress when blocked by a topoisomerase-DNA complex (62). Because of this, both mitochondrial replication and transcription may suffer as a function of altered topoisomerase function.

This suggests a mechanism for diabetic-induced declines in both mitochondrial replication as well as mitochondrial transcription, leading to a reduction in mitochondrial mRNA for translation. And this would be separate from alterations in mtDNA sequence that lead to altered protein sequence, which itself could alter formation of the mitochondria complexes (25). Any of these mechanisms could serve to alter the stoichiometry of mitochondrial proteins within separate complexes of the ETC. The consequence may be the uneven distribution of the ETC complexes leading to mitochondrial dysfunction.

Although the linkage between several anti-neoplastic agents, nuclear topoisomerases, and DNA damage is well understood, very little is known about their impact on mitochondrial topoisomerases (14, 32). Even less is known about the impact of diabetes on the mitochondrial topoisomerases. Doxorubicin, a topoisomerase poison, has been shown to accumulate in both the nucleus and the mitochondria (48). The deleterious effects of doxorubicin on the myocardium, including the mtDNA damage, are documented (5, 35). Sobuzoxane is thought to inhibit topoisomerase by trapping it in a closed clamp conformation (63). While no study has identified nuclear DNA damage as a function of sobuzoxane use, the present findings suggest caution in the use of topoisomerases inhibitors. This is evidenced by our findings that high glucose plus novobiocin led to a greater decrease in ATP production compared with high glucose alone. Hyperglycemic episodes in critically ill cancer patients are a common observation, in both known diabetics and previously undiagnosed patients, and it has a negative prognosis (3, 8, 59, 65, 69, 72).

Summary. Mitochondrial dysfunction has a significant role in the development and complications of DCM (22, 46, 51). It has been maintained that ROS is the singular source of mtDNA damage in diabetes, and the present findings are at variance with this concept. The major finding of the present study is that even mild increases in glucose presentation will compromise mitochondrial function as a result of a decline in mtDNA integrity. Separate from a direct impact of oxidative stress on mtDNA, ROS-induced alteration of mitochondrial topoisomerase activity appears to accelerate and propagate an increase in mtDNA damage. These findings are significant in that the activation/inhibition state of the mitochondrial topoisomerases will have important consequences for mitochondrial DNA integrity and the well being of the myocardium. These findings have important consequences in the management of diabetics in general and those undergoing any clinical course that targets the topoisomerases.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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