Type II diabetes increases mitochondrial DNA mutations in the left ventricle of the Goto-Kakizaki diabetic rat

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Hicks S, Labinskyy N, Piteo B, Laurent D, Mathew JE, Gupte SA, Edwards JG. Type II diabetes increases mitochondrial DNA mutations in the left ventricle of the Goto-Kakizaki diabetic rat. Am J Physiol Heart Circ Physiol 304: H903–H915, 2013. First published February 1, 2013; doi:10.1152/ajpheart.00567.2012.—Mitochondrial dysfunction has a significant role in the development of diabetic cardiomyopathy. Mitochondrial oxidant stress has been accepted as the singular cause of mitochondrial DNA (mtDNA) damage as an underlying cause of mitochondrial dysfunction. However, separate from a direct effect on mtDNA integrity, diabetic-induced increases in oxidant stress alter mitochondrial topoisomerase function to propagate mtDNA mutations as a contributor to mitochondrial dysfunction. Both glucose-challenged neonatal cardiomyocytes and the diabetic Goto-Kakizaki (GK) rat were studied. In both the GK left ventricle (LV) and in cardiomyocytes, chronically elevated glucose presentation induced a significant increase in mtDNA damage that was accompanied by decreased mitochondrial function. TTGE analysis revealed a number of base pair substitutions in the 3' end of COX3 from GK LV mtDNA that significantly altered the protein sequence. Mitochondrial topoisomerase DNA cleavage activity in isolated mitochondria was significantly increased in the GK LV compared with Wistar controls. Both hydroxycamptothecin, a topoisomerase type 1 inhibitor, and doxorubicin, a topoisomerase type 2 inhibitor, significantly exacerbated the DNA cleavage activity of isolated mitochondrial extracts indicating the presence of multiple functional topoisomerases in the mitochondria. Mitochondrial topoisomerase function was significantly altered in the presence of H2O2 suggesting that separate from a direct effect on mtDNA, oxidant stress mediated type II diabetes-induced alterations of mitochondrial topoisomerase function. These findings are significant in that the activation/inhibition state of the mitochondrial topoisomerases will have important consequences for mtDNA integrity and the well being of the diabetic myocardium.

Diabetic cardiomyopathy; type 2 diabetes; mitochondrial DNA damage; mitochondrial dysfunction

CARDIOVASCULAR DISEASE is responsible for a higher incidence of mortality in diabetics than the general population. Diabetic cardiomyopathy (DCM) is characterized by the development of a myopathy that manifests initially as diastolic dysfunction, but evolves into increased cavitary dilation and mural thinning, which is reflective of decompensated eccentric hypertrophy. DCM is considered to be independent of atherosclerosis or hypertension but is exacerbated by either (94). Mitochondrial dysfunction has long been known to have a significant role in the development and complications of DCM (32, 46, 66, 82, 92). Mitochondrial dysfunction is also associated with other pathologies including cancer, skeletal muscle disorders, and neurodegenerative diseases such as Wolfram syndrome or Leber’s hereditary optic neuropathy (LHON) (18, 36, 54, 104).

Separate from inborn errors, mitochondrial DNA (mtDNA) mutations are thought to accumulate as a function of the aging process and believed to be responsible for the decline in mitochondrial function. The mtDNA mutation rate is higher in diabetic patients than euglycemic individuals (86). Mitochondrial DNA is thought to be at a greater risk because of its close proximity to the electron transport chain (ETC) and the higher levels of reactive oxygen species (ROS) that it generates. Also mtDNA does not contain introns and any mutation is likely to alter coding sequence for one of the 13 proteins encoded by the mtDNA. In postmitotic cells, the mitochondrial genome continues to replicate about once a month (13). Diabetic-induced mtDNA mutations that are not corrected by mitochondrial DNA repair pathways are fixed in the mitochondrial genome upon replication and this may account for their greater accumulation in diabetes.

Clinically, chronic hyperglycemia and poor glycemic control are negative prognosticators for diabetic individuals. Hyperglycemia is common to many animal models of type II diabetes, including the Goto-Kakizaki (GK) rat. GK rats are nonobese, and in contrast to other models of type 2 diabetes (T2D), the GK rats do not have hyperlipidemia or hypercholesterolemia. As a result they present as a milder form of T2D that is predominantly a model of hyperglycemia.

Less clear are the underlying mechanisms leading to failure. Mitochondrial dependent reactive oxygen species (ROS) generation has been accepted as the singular cause of mtDNA damage in diabetes and other pathophysiological states. However, the mitochondria have developed defense mechanisms to handle ROS and short-term hyperglycemia has been shown to activate antioxidant defenses (39). Second, mitochondria do possess DNA repair pathways. Third, glucose is not the preferred substrate of the myocardium and diabetes is known to further decrease glycolysis in the myocardium which should lower intracellular glucose concentrations (1, 45, 64). In contrast, significant increases in pentose shunt pathway activity have been reported that may significantly increase intracellular ROS (44, 70, 105). As a result it is unclear to what extent hyperglycemia may be a factor for promoting mitochondrial dysfunction as an underlying cause of diabetic cardiomyopathy. Using the H9c2 cell line, we have previously reported 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 (80).

These findings are significant in that the functional state of the mitochondrial topoisomerases has important consequences for mtDNA integrity and the well being of the cell. So it is against this backdrop we have tested the hypothesis in a model of type 2 diabetes that chronic hyperglycemia-induced dysfunction of mitochondrial topoisomerases propagates mtDNA mutations as a contributor to mitochondrial dysfunction.

METHODS

Male euglycemic Wistar and diabetic GK rats (3–6 mo old) were used throughout this study, in addition to primary neonatal cardiomyocyte cultures. The GK animals were originally received courtesy of R. Farese (University of South Florida for Health Sciences) (115). Experimental protocols had institutional approval, and animals were maintained in accordance with the American Physiological Society's Guiding Principles in the Care and Use of Animals and the Guide for the Care and Use of Laboratory Animals (National Research Council, Revised 1996).

Cell culture. Neonatal cardiomyocytes from newborn Wistar animals were prepared using collagenase IV, as we have previously described (22, 23). Following preparation cells were plated overnight in LG-DMEM + 10% FBS + 0.1 mmol/l Brdu + 1.0 mmol/l sodium L-valine overnight before switching to the experimental media (LG-DMEM + 1% FBS + 1x NEAA + 2 mmol/l glutamine) and glucose set to 5.5, 16.5, or 33.0 mmol/l for up to 13 days. Media was changed on alternate days and where indicated drug additions made at that time. Media osmolality was balanced using mannitol.

Cellular and mitochondrial function. ATP production was measured by the CellTiter-Glo luminescent assay (Promega, Madison WI). GSH levels were measured using the GSH-Glo kit (Promega, Madison, WI). Cytochrome oxidase (complex IV) was measured by a photometric method described by Higuchi et al. (49). Superoxide was made using dihydroethidium (DHE). In brief, cells were loaded with 20 μM DHE for 30 min at 37°C, leaving one well blank as a negative control. Cells were washed twice in PBS by pelleting them at 800 g for 5 min. Cells were analyzed using a Guava EasyCyte Mini (Millipore, Billerica, MA). DHE was detected in the PM2 (red) channel and gates were set using the unstained cells and excluded cells less than 10 μm.

Mitochondrial isolation. Mitochondria were isolated by differential centrifugation as described previously (21, 80). In brief, tissues from animals were minced using fine scissors before being put into the dounce homogenizer. Cultured cells were collected in ice-cold PBS and centrifuged (300 g, 5 min at 4°C). The minced tissues or cell pellets were suspended in mitochondrial isolation buffer (250 mmol/l sucrose, 10 mmol/l Tris-Cl pH 7.5, 1 mmol/l EDTA, 1 mmol/l EGTA, 1.5 mmol/l MgCl2, 10 mmol/l KCl, 1 mmol/l DTT, 1 mmol/l PMSF, 1x protease inhibitors; Sigma P-8340) and homogenized using a dounce homogenizer (ten strokes “A” pestle and 10 strokes “B” pestle) on ice. The extracts were then subjected to successive centrifugations [1] 300 g, 5 min at 4°C times 3; 2) 1,000 g, 5 min at 4°C times 2; 3) 2,000 g 5 min at 4°C times 1; 4) 13,000 g, 10 min at 4°C times 3]. Mitochondrial pellets were resuspended in buffer (50 mmol/l Tris-Cl pH 7.5, 0.5 mmol/l EDTA, 0.5 mmol/l EGTA, 1 mmol/l DTT, 10% glycerol, 1x protease inhibitors) and lysed on ice for 30 min before protein concentration was determined by the Bradford method (Bio-Rad, Hercules CA).

mtDNA damage. Separate protocols were used to evaluate mtDNA integrity: 1) long-range PCR (LRPCR) to assess DNA strand breaks (21), 2) mitochondrial copy number, 3) reverse random polynucleotide polymorphism analysis to identify specific sequence changes, and 4) TTGE analysis to scan for sequence changes. Total DNA was extracted from cultured cardiomyocytes or 1–10 mg left ventricle using Sigma Extract-n-Amp (Sigma, St. Louis, MO) or DirectPCR (Viagen, Los Angeles, CA). The first approach used a LRPCR protocol to assess mtDNA damage by real-time QPCR using a Stratagene 3000Mx as we have previously described (21, 80). In brief, any lesion (strand breaks, base modifications, and apurinic sites) will stop a thermostable DNA polymerase capable of generating a long DNA product (21). The Ct derived from this amplification was compared with the Ct of a short PCR (SRPCR) product (150–250 bp) that is unlikely to contain any lesions. SRPCR was done using Brilliant QPCR Master Mix, while LRPCR was done using PhiUltra II Expand HiFiq DNA polymerase (Stratagene, La Jolla, CA). The primers for the LRPCR reaction were 5′-GCCCAGGCAAAACATTGTGTGTA-3′ forward and 5′-GGACTAGC CATTCACTAC-3′ reverse; other primers used were as previously described (21). Quantification of mtDNA damage and mitochondrial copy number were derived by the 2ΔCt method, from the comparison of LRPCR:SRPCR and SRPCR:β-actin, respectively. To determine if specific sites were altered, a reverse random polynucleotide polymorphism analysis (rRFLP) was used. The rationale for this approach is that any diabetic-induced alteration of an enzyme recognition sequence will blunt its restriction and increase yield upon QPCR amplification. LV DNA was prepared as described above and cut with BsrI or MboI. An aliquot of the restriction digest was taken and primers amplifying across the cytochrome B (BsrI) or D-Loop (MboI) regions of mtDNA were used in a QPCR protocol. The goal of this approach was to determine if glucose-induced damage to mtDNA resulted in nucleotide substitutions in the sequence recognized by the restriction enzyme. Quantification of the restriction site integrity was derived by the 2ΔCt method, from the comparison of cut:uncut paired samples.

Temporal temperature gradient analysis (TGGE). A PCR reaction product was amplified that included a small portion of the mitochondrial genome. The PCR products are loaded onto a denaturing polyacrylamide (5% acrylamide/9 mol/l urea) gel, and during electrophoresis the temperature of the gel is increased at a fixed rate (70 V; 20 h/48–66°C at 1°C/h). Primers were used to amplify a 543-bp fragment within the 3′ end of subunit III of cytochrome oxidase. Following electrophoresis, the bands were cutout, and the DNA extracted and used as a template for another round of PCR amplification.
mitochondrial DNA damage. Cardiomyocytes were exposed to 5.5, 16.5, or 33 mM glucose in DMEM/1% FBS for 13 days. Measurements were made as described in METHODS. mtDNA damage was assessed using the long-range PCR (LRPCR) protocol. Values are normalized to 5.5 mM glucose and are means ± SE. *P < 0.05 compared with the 5.5 mM control.

this was followed by significant increases in MitoSox fluorescence (Fig. 1). In the diabetic heart, glucose usage has been shown to shift away from glycolysis and toward an increased flux through the pentose phosphate pathway (1, 8, 70, 84, 105, 118). In agreement with those reports, we observed that 13 days of elevated glucose presentation resulted in a significant increase in cardiomyocyte glucose-6-phosphate dehydrogenase (G6Pdh) activity (Fig. 2A) and to a lesser extent in H9262 cells (5.5 mM glucose, 10 ± 5.0%; 33 mM glucose, 115.3 ± 4.8%; P < 0.05). The inclusion of dehydroepiandrosterone (DHEA), an inhibitor of the G6Pdh, blocked glucose-induced increases in cytosolic ROS (Fig. 2B). DHEA appeared to have a protective effect and blocked glucose-induced mtDNA damage (Fig. 2C) and restored cardiomyocyte ATP production (Fig. 2D). Similar to DHEA, 6-aminonicotinamide, a G6Pdh competitive inhibitor, restored ATP production in hyperglycemic cardiomyocytes (data not shown). Left ventricle (LV) G6Pdh activity was significantly increased in the diabetic GK LV compared with the Wistar LV (Fig. 3A). G6Pdh is the first and rate limiting step in the pentose shunt pathway, and increased NADPH generation is a product of this reaction. Previously, we have reported that NADPH oxidase-dependent superoxide production was significantly increased in the aorta of GK rats (42). Similar to that finding, we observed a significant increase in myocardial NADPH-driven chemiluminescence generation (Fig. 3B) as well as H2O2 production (Fig. 3C). In contrast, NADH-driven chemiluminescence generation (Fig. 3B) was significantly depressed. These findings suggest that extramitochondrial superoxide generation provided a significant source of oxidant stress in the diabetic heart.

Mitochondrial dysfunction has been observed in both animal models of diabetes and in diabetic patients (86). In agreement with those observations, cytochrome oxidase activity was significantly decreased in LV extracts as well as in both the SSF and IMF mitochondrial fractions (Fig. 4A). In conjunction with this, we observed significant decreases in cytochrome oxidase subunit IV (nuclear encoded) and subunit III (mitochondrial encoded) from the GK LV (Fig. 4B).

Nomiyama et al. (86) reported that the mtDNA mutation rate was significantly higher in diabetic patients than in healthy...
individuals. Similarly, in both H9c2 cells (80) and neonatal cardiomyocytes (Fig. 1), mtDNA damage was significantly increased as a function of elevated glucose presentation. In vivo a significant increase in mtDNA damage was also observed in the diabetic GK LV (Fig. 5 A), without a change in mitochondrial copy number (Fig. 5 B). Although mitochondrial deletions have been reported in models of aging, we did not see any consistent evidence of deletions in the GK LV (Fig. 5 C). Using a reverse RFLP approach with the restriction endonucleases Bsr I (the cytochrome B region) or Mbo I (D-Loop region), we did not find any evidence of diabetic-induced sequence specific alterations (data not shown).

DNA damage may take several forms including nucleotide substitution, insertions, deletions, or breakpoints as our LRPCR approach has focused on. Several methodologies have been applied in an attempt to permit comprehensive scanning of the mitochondrial genome. These include RFLP, SSCP, allelic specific oligonucleotide dot blot hybridization, or denaturing gradient gel electrophoresis. The LRPCR approach has focused on mtDNA damage from increased glucose exposure. mtDNA damage was assessed using the (LRPCR protocol). DHEA restored ATP production in glucose-challenged cardiomyocytes. Values are normalized to 5.5 mM glucose or 5.5 mM vehicle and are means ± SE. *P < 0.05 compared with the 5.5 mM glucose control; #P < 0.05 compared with 33 mM glucose/vehicle.

Fig. 2. Elevated glucose increased glucose-6-phosphate activity to increase cytosolic reactive oxygen species (ROS) and induce mtDNA damage. Cardiomyocytes were maintained in DMEM/1% FBS and exposed to 5.5, 16.5, or 33 mM glucose for 13 days. A: elevated glucose increased glucose-6-phosphate dehydrogenase (G6PdH) activity in cardiomyocytes. B: dehydroepiandrosterone (DHEA) counteracted glucose-induced increases in cytosolic ROS. Cells were treated with 20 μM DHE for 30 min before being washed, and cells greater than 10 μm were counted in a Guava EasyCyte Cytometer. C: DHEA prevented mtDNA damage from increased glucose exposure. mtDNA damage was assessed using the (LRPCR protocol). D: DHEA restored ATP production in glucose-challenged cardiomyocytes. Values are normalized to 5.5 mM glucose or 5.5 mM vehicle and are means ± SE. *P < 0.05 compared with the 5.5 mM glucose control; #P < 0.05 compared with 33 mM glucose/vehicle.

Fig. 3. A: diabetes increased glucose-6-phosphate dehydrogenase activity in the diabetic left ventricle (LV). B: diabetes increased NADPH-driven chemiluminescence, but not NADH-driven chemiluminescence, in the diabetic LV. C: diabetes increased LV H2O2 production. LV extracts were prepared and analyzed for activity as described in METHODS. G6Pdh was analyzed following the protocol of Serpillon et al. (105), while superoxide was determined by a lucigenin chemiluminescence protocol (40). GK, Goto-Kakizaki. Values are mean ± SE. *P < 0.05, #P < 0.10 compared with the respective control.
dient gel electrophoresis (DGGE) analyses. More recently, tem-
poral temperature gradient electrophoresis (TTGE) has been de-
veloped. A small section of the mitochondrial genome is
amplified and during electrophoresis the temperature of the gel is
increased at a fixed rate. This brings about strand dissociation and
allows for resolution of DNA fragments that differ by as little as
a single nucleotide substitution. This approach permits screening
for either homoplasmic or heteroplasmic mutations (11, 85).

Using a TTGE analysis, no changes in mtDNA sequence were
observed in the six Wistar LV samples analyzed. In contrast, four
of six GK LV exhibited mtDNA mutations within the COX 3
subunit region. Three of four GK LV samples had mutations that
included base pair substitutions or insertions which altered the 3'
end of the COX3 coding sequence resulting in early termination.
In a fourth animal (GK355), a conservative single amino acid
substitution was observed exchanging a glutamine to threonine,
both of which are polar uncharged amino acids. Table 1 illustrates
the translated consequences of the mtDNA changes for the cyto-
chrome oxidase subunit III protein sequence.

Topoisomerases have complex reaction mechanisms in
which the enzyme covalently binds to the DNA and induces
strand breakage before DNA torsion stress is relieved. This is
followed by strand religation of the DNA before the enzyme
disengages. Relief of torsional stress is demonstrated in vitro
by the DNA relaxation assay using supercoiled plasmid DNA.
As shown in Fig. 6A, the mitochondrial extracts generated the
same isopane pattern of relaxation as purified nuclear topo-

sisomerase 1 (lane 4). When isolated mitochondria were passed
through a G200 Sephadex column, DNA cleavage assay of the
size-separated fractions found a significant increase in DNA
cleavage activity in the 150- to 160-kDa fractions correspond-
ing to mitochondrial topoisomerase IIβ (data not shown).
Many topoisomerase inhibitors increase DNA strand breaks
without a concomitant increase in ligation as detected by the
DNA cleavage activity. When isolated mitochondria were
incubated in the presence of the topoisomerase inhibitors
hydroxycamptothecin (type 1) or doxorubicin (type 2), both
drugs enhanced myocardial mitochondrial topoisomerase DNA

Fig. 4. Diabetes decreased cytochrome oxidase activity and subunit 3 and 4 expression in the diabetic LV. A: cytochrome oxidase activity was determined by
oxidation of reduced cytochrome c as described in METHODS (98). SSF, subsarcolemmal fraction; IMF, intermyofibrillar fraction. B: quantification of subunit 3
and 4 in LV. Values are means ± SE: *P < 0.05, #P < 0.10 compared with Wistar control.

Fig. 5. A: diabetes damages mtDNA in GK left ventricle compared with Wistar LV. mtDNA damage was assessed using the LRPCR protocol as described in
METHODS. B: diabetes did not influence LV mitochondrial copy number. mtDNA copy number by PCR and is the ratio of mtDNA:actin DNA. Values are
normalized to Wistar control and are means ± SE *P < 0.05. C: representative LRPCR mtDNA products from Wistar and GK LV.
cleavage activity (Fig. 6, B and C). Diabetes induced significant
clearances in mitochondrial topoisomerase DNA cleavage
activity in mitochondrial extracts derived from the diabetic
GV. Although not specific for any of the three unique mitochondrial topoisomer-
ases, the average DNA cleavage suggests that mitochondrial
topoisomerase function was collectivly altered by diabetes.

Cai et al. (7) reported an increase in cytosolic oxidant stress
soon after exposure to increased glucose. Li et al. (68) reported
that oxidant stress altered nuclear topoisomerase I function to
depress the re-ligation step. This suggests that with diabetes
the activation/inhibition state of the mitochondrial topoisomerases
will have important consequences for mtDNA integrity and main-
taining myocardial function.

The GK rat is a nonobese model of type II diabetes that is
essentially a model of hyperglycemia without significant hy-
perlipidemia or hypercholesterolemia. The GK rats develop
a Fenton reaction participated since the reactions were per-
formed in the presence of the iron chelator, EDTA. When
mitochondrial extracts were incubated in the presence of 60
µM H2O2, mitochondrial topoisomerase DNA cleavage was
significantly increased beyond that of cleavage in the absence
of H2O2 (Fig. 7A). Topoisomerase IIB has known phosphory-
lation state and that phosphorylation of nuclear topoisomerase
IIB significantly altered its activity (73, 76, 129). These se-
quences are conserved in the mitochondrial topoisomerase IIB.
Using alkaline phosphatase to modify the phosphorylation state
of mitochondrial topoisomerases, we observed that DNA
cleavage activity was significantly decreased compared with
vehicle-treated mitochondria (Fig. 7B). This suggests that
phosphorylation state may also regulate mitochondrial topo-

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DISCUSSION

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The GK rat is a nonobese model of type II diabetes that is
essentially a model of hyperglycemia without significant hy-
perlipidemia or hypercholesterolemia. The GK rats develop
elevated fasting glucose, exhibit decreased glucose intolerance,
and increased HbA1c levels at an early age (37, 40, 42, 124,
127). GK rats present with many diabetes-related complica-
tions observed in human diabetic patients, including reduced
nerve conduction velocity and progressive renal involvement
(53, 83, 93, 115, 124 –126). D’Souza reported in the GK
ventricle an increase in wall thickness that was associated with
increased myocyte diameter and collagen matrix proliferation
as well as increased expression of the fetal gene pattern,
characteristic of an adaptive response to overload (19). Al-
though not hypertensive, the GK rats exhibit a small increase in
MAP and systolic blood pressure compared with their Wistar

mitochondrial dysfunction. Alone 60 prolonged by an oxidant-induced alteration of mitochondrial
exaggerated hyperglycemic response to a meal may be further
depress the re-ligation step. This suggests that with diabetes
that oxidant stress altered nuclear topoisomerase 1 function to
soon after exposure to increased glucose. Li et al. (68) reported
actively phosphorylated and underlined sequences are the altered amino sequence resulting
from the altered mtDNA sequence.

Table 1. TTGE analysis of cytochrome oxidase subunit III

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Fasting Glucose, mol/l</th>
<th>Total Errors in COX III</th>
<th>Average Errors per Animal</th>
<th>No. of Animals With Errors</th>
</tr>
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<tbody>
<tr>
<td>Wistar</td>
<td>6</td>
<td>4.17 ± 0.11</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GK</td>
<td>6</td>
<td>7.11 ± 0.44</td>
<td>13</td>
<td>2.2</td>
<td>4/6</td>
</tr>
</tbody>
</table>

Wild type

MTHQTHAYHMNVPSWPWTGALSALLLTSGILVWFHYNSTILLSLGLTNILTMYQWWRDIREGTYGHHHTPIVQGLRY
GMILFIVSEVFAGFFWAYHSLLVPHTLDLGCCWPPTITPLNPVEPLNTSVALASGVSTWAHSLMEGNRNHMQGPTNHSL

Goto-Kakizaki:

GK312

MTHQTHAYHMNVPSWPWTGALSALLLTSGILVWFHYNSTILLSLGLTNILTMYQWWRDIREGTYGHHHTPIVQGLRY
GMILFIVSEVFAGFFWAYHSLLVPHTLDLGCCWPPTITPLNPVEPLNTSVALASGVSTWAHSLMEGNRNHMQLTILLG
LYFTILQASEKMFRIIFLY

GK318

MTHQTHAYHMNVPSWPWTGALSALLLTSGILVWFHYNSTILLSLGLTNILTMYQWWRDIREGTYGHHHTPIVQGLRY
GMILFIVSEVFAGFFWAYHSLLVPHTLDLGCCWPPTITPLNPVEPLNTSVALASGVSTWAHSLMEGNRNHMQALLITILLG
YFTILQASEYFETSISDGIYSTFFMATGFGHLHIVGSTFLYCLRRTKIKLHIKTSFRIWSSMMLTIER

GK355

MTHQTHAYHMNVPSWPWTGALSALLLTSGILVWFHYNSTILLSLGLTNILTMYQWWRDIREGTYGHHHTPIVQGLRY
GMILFIVSEVFAGFFWAYHSLLVPHTLDLGCCWPPTITPLNPVEPLNTSVALASGVSTWAHSLMEGNRNHMQALLITILLG
YFTILQASEYFETSISDGIYSTFFMATGFGHLHIVGSTFLYCLRRLKHFSTKHFSTHFGFEEAAYWYHFVDVWFLYVSITYWYG
SYS

GK, Goto-Kakizaki diabetic rat; TTGE, temporal temperature gradient analysis. Italicized and underlined sequences are the altered amino sequence resulting from the altered mtDNA sequence.
controls (12). By echocardiography, we did not observe significant differences in the GK fractional shortening or ejection fraction under basal conditions (data not shown). However, El-Omar et al. (26) did observe that in response to a hypoxic challenge, diastolic function was significantly altered in the GK hearts, a finding consistent with DCM.

Mitochondrial-dependent ROS generation has been accepted as the singular cause of mtDNA damage in diabetes and other pathophysiological states. Several lines of evidence point toward a glucose-induced increase in mitochondrial ROS including ESR studies, image analysis using MitoSox, as well as studies using yellow fluorescent protein (cpYFP) to measure superoxide flashes (4, 7, 47, 48, 74, 78, 119). A common limitation is that they only examined changes as an early event. We and others that have examined a longer time frame have observed changes in mitochondrial ROS to be a transient event in response to chronically increased glucose or palmitate presentation. Further, Herlein et al. have argued the position that in mild diabetes or prediabetes, mitochondrial superoxide may not be elevated in contrast to its decided presence in more severe diabetic states (6, 48, 78). These events point toward other pathways participating in the prediabetic state or mildly diabetic state to propagate mitochondrial dysfunction.

The role of radical oxygen species (ROS) in cellular metabolism is evolving. Historically perceived as a by-product of aerobic metabolism, the cell’s ability to generate specific different oxidant species indicates a significant and useful purpose (9, 15). More recently, this has taken the concept of redox signaling and the appreciation that the cell is not a dilute solution but a crowded highly organized space (9, 33, 114). Compartmentalization of ROS is both by physical separation as well as activation of distinct enzyme complexes.

**Fig. 6.** A: DNA relaxation of supercoiled plasmid DNA. Lane 1, no extract; lane 2, 1.0 µg mitochondrial extract; lane 3, 0.5 µg mitochondrial extract; lane 4, 2.5 U purified topoisomerase 1; lane 5, linearized pBR322. B: 10 µM hydroxycapecitabine exacerbated DNA cleavage of LV mitochondrial extracts. Values are means ± SE and are normalized to no mitochondria (No Mito) control. *P < 0.05 compared with No Mito control; #P < 0.05 compared with mitochondria alone (+Mito). C: 0.5 mM doxorubicin exacerbated DNA cleavage of LV mitochondrial extracts. Values are means ± SE and are normalized to No Mito. D: diabetes increased myocardial mitochondrial topoisomerase DNA cleavage activity. Myocardial mitochondrial extracts were incubated with linear mtDNA for 30 min before being electrophoresed, and cleavage products were quantified by band intensity. Values are means ± SE and are normalized to Wistar DNA cleavage. *P < 0.05 compared with Wistar LV mitochondrial extract.

**Fig. 7.** Regulation of myocardial mitochondrial topoisomerase activity. A: H2O2 increases mitochondrial topoisomerase-dependent DNA cleavage. Mitochondria were preincubated in the absence or presence of 60 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. B: dephosphorylation alters mitochondrial topoisomerase DNA cleavage activity. No Mito: no extract; + Mito: untreated mitochondria; CIP trt, mitochondria treated with 1 U calf intestinal phosphatase for 30 min at 37°C before DNA was added. Values are means ± SE and are normalized to no Mito or no Mito/H2O; *P < 0.05 compared with No Mito; #P < 0.05 compared with untreated mitochondria.
the mitochondria, the NADPH oxidase system and NOS enzymes generate a significant portion of cellular ROS, while other molecules serve to act as buffers. Although clearly shown to have a destructive effect, a concentration-dependent biphasic effect of ROS is apparent within the myocardium. Low concentrations of ROS appear to serve as signaling molecules, while higher levels propagate a destructive outcome (10, 60, 65). The threshold for this biphasic effect appears in part to be dependent on the oxidant buffering capacity of the cell, as decreases in glutathione levels appear to lower the threshold for stress-induced damage within the cell (28, 78, 88, 89). GSH, an oxidant scavenger, is depleted by diabetes across different tissues and models of diabetes (5, 27, 34, 61, 106, 107, 123). Consistent with those reports we observed significant decreases in GSH as an early change that preceded increases in mitochondrial ROS or mtDNA damage. Although increased G6PdH activity might be thought to increase GSH levels, that is not the case here and suggests that depletion of oxidant buffers may be an early step in the downward spiral of mitochondrial function.

Diabetes increases flux through the pentose shunt pathway in the heart, and this may be a significant source of oxidant stress within the cytosol (43, 57, 105). Glucose-6-phosphate dehydrogenase (G6PdH) is rate limiting in this pathway and in the present study its inhibition reduced glucose-induced mtDNA damage and restored cellular ATP production. In a STZ model of diabetes, Ozdemir et al. (90) also observed significant increases in G6PdH and 6-PGD activity and these changes were reversed by treatment with the ANG II antagonist, candesartan-cilexetil. Many papers have shown that angiotensin blockade counteracts the diabetic state (3, 29, 40, 87, 90, 95, 100). Not just an endocrine but also an autocrine, elevated glucose increased ANG II expression and release from cardiomyocytes (31). Ricci et al. (99) reported an increase in mtDNA damage in response to 1 h of 1 mM ANG II in cultured cardiomyocytes. However, the increase in mtDNA damage and drop in mitochondrial ΔΨm lagged behind the rapid rise in cytosolic superoxide levels but preceded detectable increases in mitochondrial superoxide. This suggests that mtDNA damage and mitochondrial dysfunction occurred by activation of an alternative pathway or production of an alternative oxidant species. Aside from glucose-6-phosphate, the major reaction product of G6PdH is NADPH, the substrate for NADPH oxidase. Diabetes increases myocardial Nox4 expression, and its localization to the outer membrane of the mitochondria is significant (4, 38, 75). Unlike Nox1, Nox4 does not require p47 or p67 for activation, and biochemical studies suggest that it preferentially generates H2O2 over superoxide (16, 79, 108). Block et al. (4) demonstrated in mesangial cells that a siNox4 probe abrogated glucose-induced increased superoxide generation. Both in the diabetic heart and in cultured cardiomyocytes N-acetyl-l-cysteine (NAC), a cytosolic localized antioxidant, has been reported to decrease oxidative stress markers (30, 59). These reports are all consistent with our findings that NADPH oxidase activity and cytosolic H2O2 activity were increased in the GK hearts, while NADH-driven oxidase activity was not. And it argues against mitochondrial-driven NADH oxidase as being the singular or even predominant source of superoxide in the GK heart. Collectively these findings suggest that a substantial increase in cytosolic-derived ROS that has access to the myocardial mitochondrial compartment may be the predominant source of oxidative stress in this hyperglycemic model of diabetes.

Diabetes induces many changes that are cell and tissue specific. While cardiomyocyte glycolysis is decreased with diabetes, the opposite is observed in endothelial cells (96). The results in neonatal cardiomyocytes and in diabetic hearts were similar to our earlier findings in the H9c2 cell line; chronic increases in glucose presentation led to a degradation of mitochondrial function. In that study, individual ETC complexes containing mtDNA-encoded sequences were compromised by hyperglycemia, while the Complex II (a mitochondrial complex that is wholly nuclear encoded) was not (80). Separate from hyperglycemia, chronically elevated palmitate presentation also significantly decreased ATP levels (97). Our findings are also similar to streptozotocin-induced diabetes, in which significant decreases in the levels of the mitochondrialry encoded COX I or IV were observed (55). In the present study mitochondrial dysfunction was linked to the degradation of the ETC proteins.

The concept of increased mtDNA replication as a means to overcome mtDNA mutations has been advanced by a number of studies (17, 25, 52, 101, 113). However it remains unclear if this is a stable or transient event, and whether it expands or dilutes the mtDNA mutation pool. Recently Tewari et al. (110a) demonstrated that elevated glucose presentation or diabetes decreased expression of the nuclear encoded mitochondrial transcriptional machinery and that its restoration by relief of oxidant stress or by overexpression of POLG1 ameliorated mitochondrial dysfunction. Their data suggest that decreased mtDNA transcription was a function of lower expression rather than mtDNA damage within the D-Loop region. Red ragged fiber syndrome is a function of mtDNA mutations and increased copy numbers have been observed in aged muscle (36). This is thought to be an attempt by the cell to compensate for the loss of ATP generation capability. Santos et al. (102) observed that the increase in copy number was a transient event, being elevated at 2 mo but then depressed at 6 and 12 mo. In the present study we did not observe significant changes in mtDNA copy number in the GK heart. Our use of the LRPCR protocol identified DNA strand breaks or other obstructions that blocked progression of the DNA polymerases. That the Pfu DNA polymerase used in the LRPCR analysis was unable to amplify across DNA breaks has some important consequences. Both mammalian DNA and RNA polymerases are unable to read across DNA breaks (109). As well, these polymerases are also unable to progress when blocked by a topoisoasmerase-DNA complex (109). Thus both replication and transcription may suffer as a function of mtDNA damage and altered topoisoasmerase activity. Thus the differences in reported copy numbers among these studies may lie in the different models used; the STZ is a severe form of type 1 diabetes vs. GK, which is a mild form of type 2 diabetes.

The reverse RFLP approach used in the present study determined that specific sequences were unaltered by diabetes. Since this approach only scans 6 bp segments, it is likely that this approach did not have sufficient sensitivity to identify significant damage. The TTGG protocol scanned across distinct regions of mtDNA and identified point mutations that altered the primary mtDNA sequence. Importantly, we did not observe a consistent pattern of mutations or deletions. The data suggest that diabetic induced damage did not target specific sequences of the mtDNA.
and that damage occurred in a random rather than a patterned manner. Others have reported the presence of hepatic mtDNA deletions from alcoholic patients (77). As well, the lack of deletions is different for those studies that have examined the effects of aging on mtDNA states (36, 117). Collectively, these findings suggest that the underlying mechanisms for diabetic induction of mtDNA damage might differ from other pathophysiological states.

The changes in mtDNA sequence varied but each significantly altered the coding sequence resulting in early termination or mutation of the C-terminal region. Although our only examined the C-terminal region of COX III, the results are likely to be reflective across much of the mtDNA. The impact of altered mtDNA primary sequence has been more dramatically demonstrated by the mtDNA-Pol\textsuperscript{def} transgenic mice in which the mtDNA polymerase lacked “proofreading” capability. In those mice, cardiac cells accumulated mtDNA mutations at a rate of more than 20-fold compared with controls, had a significant increase in apoptosis, and presented with significant heart failure (130, 131). Despite this, there was not a significant change in mitochondrial function; the P/O ratio and respiratory control index were similar in mtDNA-Pol\textsuperscript{def} and controls. Interestingly, markers for ROS were not increased, suggesting that oxidative stress was not an obligate mediator of mtDNA mutations. Although not diabetic, this model suggests that any increase in mtDNA mutations may serve as an underlying cause of mitochondrial dysfunction. Others have also reported that ROS production and oxidative damage were not increased in different tissues of mice with depletion (117) or point mutations of mtDNA (50, 58, 111). An alternative conclusion to those studies was that the accelerated apoptosis rid the heart of dysfunctional cells and those remaining cells did not yet have demonstrable ROS. Interestingly, the mtDNA-Pol\textsuperscript{def} mice lacked significant mtDNA deletions, to which the authors argue that point mutations and not deletions are involved in the accelerated defective phenotype (20). They observed decreases in the presence of fully assembled Complex I and IV but not Complex II and our results concur. Separate from those studies, two papers examined the impact of mtDNA mutations on OXPHOS assembly (35, 91). A mutation in cytochrome b (A15533G) was observed in a patient presenting with lactic acidosis and mild mental decay. Mimicking the mutation in transmutational cybrids resulted in significant alterations in the rate of Complex assembly (35). Using a similar approach and examining mutations common to LHON (Leber’s hereditary optic neuropathy), Pello et al. observed significant differences in the rates of ETC complex assembly (91). Another considerable problem generated by point mutations is the interdependency of the mitochondrial complexes not only for their assembly but also stability within the mitochondrial membrane (2, 20, 62, 112). Collectively, these studies point to the critical role that mtDNA fidelity has on the mitochondrial function.

In postmitotic cells, the mitochondrial genome is thought to replicate about once per month and resolving the topology of mtDNA replication would be almost insurmountable in the absence of the mitochondrial topoisomerases (13). Three unique topoisomerases have been demonstrated in the mitochondria; topoisomerase IIβ (a type II), mtTOP1 (a type IB), and TOP3α (a type IA) (72, 80, 120, 132). mtTOP1 is a paralog of the nuclear isoforms and likely arose by a gene duplication event. mtTOP1 has a lower DNA binding affinity, and it has been suggested that this may serve to relieve supercoiling of newly formed RNA transcripts rather than participate in mtDNA replication (14, 103). We have previously reported that immunoprecipitation of mtTOP1 topoisomerases from isolated mitochondrial preparations decreased DNA cleavage activity indicating the presence of functional mtTOP1 in the mitochondria (80). Wu et al. (122) reported that in Drosophila knockout of the mtTOP3α gene resulted in a decline of mtDNA suggesting that it participates in the maintenance of the mitochondrial genome. The functional role of mitochondrial topoisomerase IIβ also remains unclear; however, it has been suggested to participate in decatenating newly synthesized mtDNA circles (72). Because the topoisomerase IIβ produces double strand breaks, it may also be responsible for the deletions observed in some tissues but not in others. In the present study, we have demonstrated that diabetes significantly increased the DNA cleavage activity of the mitochondrial topoisomerases. The data indicate the participation of all three proteins in mtDNA cleavage but at present it is not possible to determine if one mitochondrial topoisomerase dominates the diabetic-induced increases in myocardial mtDNA damage.

Several posttranslational modifications exist that may modulate enzyme function, and very little is known about regulation of mitochondrial topoisomerases. Most of what is suspected has been inferred from studies of nuclear topoisomerases. In addition to changes in glycolysis and the pentose shunt pathway function, hyperglycemia is also thought to alter the activity of the hexosamine pathway. GlcNacylation is increased by hyperglycemia/diabetes and a mitochondrially directed splice variant of O-GlcNacylation has been reported (51, 71), suggesting that this pathway may also be important in the mitochondrion. In STZ diabetes, increased TOP1 GlcNacylation was associated with a decrease in nuclear TOP1 DNA relaxation activity (67). Both protein kinase C and casein kinase I δ/ε activity have been shown to modulate DNA cleavage activity of nuclear topoisomerase IIα (41, 128). Incubation of alkaline phosphate (to promote dephosphorylation) decreased nuclear topoisomerase I relaxation of supercoiled (SC) DNA (110). Both Top1 and mtTop1 have a common phosphorylated site Tyr\textsuperscript{288} within the core region of the enzyme, and modification at this site has been shown to alter camptothecin sensitivity (129). Others have shown that Tyr\textsuperscript{273} phosphorylation alters its preference for supercoiled or relaxed DNA (73, 76). The present study makes similar observations in that we observed that phosphatase treatment decreased DNA cleavage activity of the mitochondrial topoisomerases, suggesting a significant role for posttranslational regulation of mitochondrial topoisomerase function.

Summary

Studies examining mitochondrial dysfunction and mtDNA damage have focused on elevated mitochondrial oxidant stress as a singular cause for diabetic cardiomyopathy as well as other pathologies such as alcoholism, cancer, neurodegeneration, and radiation-induced mitochondrial dysfunction. However, antioxidant therapy studies have yielded results that range from disappointing to a potentially detrimental effect of antioxidants (24, 69, 81, 116). Other approaches that raise or lower mitochondrial antioxidant capacity have also yielded conflicting...
results (63, 121). These findings point to a more complex interaction of ROS within the mitochondria and suggest that alternative pathways may mediate the effect of ROS on mitochondria and mtDNA integrity. Mitochondria do have DNA repair capability, and our findings suggest that with diabetes or chronic hyperglycemia there may be limits to functional recovery. In part this may be due to exhaustion of the endogenous oxidant 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 as a result of a direct attack of ROS on mtDNA. Our finding of only transient increases in mitochondrial ROS levels in the H9c2 cell line suggested activation of alternative processes (80). Separate from a direct impact of oxidative stress on mtDNA, ROS-induced alteration of myocardial mitochondrial topoisomerase activity accelerated and propagated increases in mtDNA damage. These observations also now include the diabetic-induced increases in mitochondrial topoisomerase activity derived from the diabetic GK left ventricle. These findings are significant in that the activation/inhibition state of the mitochondrial topoisomerases will have important consequences for mtDNA integrity and the well being of the myocardium. Topoisomerases are the focal point for many antibotic and antineoplastic reagents, and their regulation is central to the clinical management of different diseases. Understanding the regulation of the mitochondrial topoisomerases is critical for protection of myocardial mtDNA, not only for the management of diabetes but also for the many other clinical treatments that target the topoisomerases.

Innovation

Mitochondrial oxidant stress has been widely accepted as the singular cause of diabetic-induced mtDNA damage leading to mitochondrial dysfunction. The present manuscript takes exception to this idea on two levels. First, separate from a direct impact of oxidative stress on mtDNA, ROS-induced alteration of myocardial mitochondrial topoisomerase function accelerated and propagated increases in mtDNA damage. Second, in this model of type II diabetes, the cytosol generates significant oxidant stress separate from and in addition to the mitochondrial sources.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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