Mitochondrial transcription factor A regulation of mitochondrial degeneration in experimental diabetic neuropathy

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Chandrasekaran K, Anjaneyulu M, Inoue T, Choi J, Sagi AR, Chen C, Ide T, Russell JW. Mitochondrial transcription factor A regulation of mitochondrial degeneration in experimental diabetic neuropathy. Am J Physiol Endocrinol Metab 309: E132–E141, 2015. First published May 5, 2015; doi:10.1152/ajpendo.00620.2014.—Oxidative stress-induced mitochondrial dysfunction and mitochondrial DNA (mtDNA) damage in peripheral neurons is considered to be important in the development of diabetic neuropathy. Mitochondrial transcription factor A (TFAM) regulates mitochondrial DNA replication and transcription. We studied whether overexpression of TFAM reverses experimental peripheral diabetic neuropathy using TFAM transgenic mice (TFAM Tg) that express human TFAM (hTFAM). Levels of mouse mtDNA and the total TFAM (mouse TFAM + hTFAM) in the dorsal root ganglion (DRG) increased by approximately twofold in the TFAM Tg mice compared with control (WT) mice. WT and TFAM Tg mice were made diabetic by the administration of streptozotocin. Neuropathy end points were motor and sensory nerve conduction velocities, mechanical allodynia, thermal nociception, and intraepidermal nerve fiber density (IENFD). In the DRG neurons, mtDNA copy number and damage to mtDNA were quantified by qPCR, and TFAM levels were measured by Western blot. Mice with 16-wk duration of diabetes developed motor and sensory nerve conduction deficits, behavioral deficits, and increased metabolic influx into mitochondria increases respiratory activity (15). In contrast, age-related depletion of mtDNA in peripheral neurons from diabetic rodents exhibit reduced respiratory chain activity, and mitochondrial DNA (mtDNA) levels are decreased in chronic diabetic neuropathy (45, 56). In contrast, factors that promote mitochondrial regeneration would rescue impaired mitochondria and may be protective in diabetic neuropathy. Mitochondrial biogenesis requires the participation of two genetic systems, nuclear DNA (nDNA) encoding the majority of proteins that are transported to mitochondria and the mitochondrial genomic system (19, 20, 25, 26, 28, 33). mtDNA contains two promoters, the light- (LSP) and heavy-strand promoters (HSP), from which transcripts are produced and then processed to yield the individual mtRNAs encoding 13 subunits of the oxidative phosphorylation system, ribosomal, and transfer RNAs (4, 6, 14, 26). Mitochondrial transcription factor A (TFAM) is an nDNA-encoded protein that binds upstream of the LSP and HSP of mtDNA and promotes replication and transcription of mtDNA (8, 27, 53). TFAM is essential for maintenance of mtDNA and for cell survival. Homozygous knockout of TFAM is embryonic lethal (29). In contrast, tissue-specific knockout mice survive and have been used to study the role of TFAM in disease models (52). Mice with TFAM knockout in pancreatic β-cells develop diabetes from the age of 5 wk and display mtDNA depletion, deficient oxidative phosphorylation, and abnormal-appearing mitochondria in islets at the ages of 7–9 wk (50). Overexpression of TFAM in mice protects against delayed neuronal death due to forebrain transient ischemia (23), improves working memory (21), and protects mitochondria against β-amyloid-induced oxidative damage (59). Therefore, there is a rationale to test the effect of TFAM overexpression in diabetic neuropathy.

TFAM plays a dual role. First, TFAM maintains mtDNA copy number by promoting mtDNA replication, which is essential for preservation of mitochondrial function (8). mtDNA copy number correlates with mitochondrial gene expression levels as well as with mitochondrial respiratory activity (15). In contrast, age-related depletion of mtDNA in human islets contributes to decreased mitochondrial function and risk of type 2 diabetes (38). The second role of TFAM is structural. TFAM wraps mtDNA entirely to form a nucleoid structure similar to histones in the nucleosome (1, 39, 58) that may also protect mtDNA against ROS (9). Recent results show that it is important to maintain the ratio of TFAM to mtDNA within a narrow range, since small TFAM variations may affect transcription and mtDNA replication (16). A small increase in TFAM levels in vivo (≈2-fold) leads to a proportional increase in mtDNA.

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The pathogenic event proposed in diabetic neuropathy is that chronic diabetes causes an increase in the generation of ROS, resulting in increased mtDNA mutations, and loss of mtDNA. These changes in turn cause impaired mitochondrial respiratory function (11, 17), loss of cellular energy, reduced ATP, and neuronal degeneration. We tested whether overexpression of TFAM would upregulate the mtDNA levels and protect against peripheral neuropathy.

MATERIALS AND METHODS

Generation of TFAM transgenic mice. TFAM transgenic (Tg) mice were generated as described previously (24). In brief, a modified chicken β-actin promoter with cytomegalovirus enhancer-driven human TFAM (hTFAM) cDNA construct was microinjected into the pronuclei of fertilized eggs of C57BL/6 mice. Founder lines were identified by the presence of the hTFAM cDNA in the tail DNA (24). Human TFAM-positive Tg mice were mated to C57BL/6 wild-type (WT) mice to generate WT and heterozygous Tg mice. WT and TFAM Tg mice were used at 10 to 13 wk of age.

Animals. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Maryland and the Veterans Affairs Maryland Health Care System. Male C57Bl/6j mice were fed standard chow (no. 7001; Harlan-Teklad, Madison, WI) with a 12:12-h light-dark cycle and free access to food and water. At 3 mo of age, diabetes was induced via serial intraperitoneal injections of streptozotocin (STZ) over 6 days, following our published procedure (11). Mice having blood glucose levels of 300 mg/dl (16.7 mM) or greater were considered to be diabetic. Age-matched nondiabetic control male mice were injected with vehicle instead of STZ. WT and TFAM Tg mice were tested at 6 and 16 wk after the induction of diabetes. There were four groups of mice at each time point: WT nondiabetic (n = 30), WT diabetic (n = 60), TFAM Tg nondiabetic (n = 30), and TFAM Tg diabetic (n = 60).

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Evaluation of nerve conduction velocity, von Frey sensory testing, thermal latency, and intraepidermal nerve fiber density. For phenotyping neuropathy in animals, the guidelines provided by the diabetic neuropathy study group of the European Association for the Study of Diabetes (Neurodiab) were followed (7). For nerve conduction studies, mice were anesthetized with either ketamine and xylazine or isoflurane. Thermal support was provided, and tail and sciatic-peroneal nerve conduction studies were performed as described previously (11, 57). Tail and limb temperatures were maintained at 32–33°C. Tail orthodromic sensory responses were obtained using low-intensity, long-duration supramaximal stimulation, and averaging of the responses until the baseline and the recording were stable.

Mechanical allodynia was assessed using Somedic von Frey mono- filaments, using the Semmes-Weinstein series (Somedic Sales) as described in detail (11). Ordinal numbers >4 were applied gently on the fat pad of both plantar heels until the hair started to bend and maintained for ~2 s. The threshold was defined as the minimal bending force of the thinnest filament sensed by the mouse in an ascending and descending series of applications. A withdrawal response is considered valid only if the hindpaw is completely removed from the platform. Hargreaves’ test was used to test thermal nociception, which assesses small nerve fiber function. Mice were left in a multiple animal enclosure cage (Harvard Apparatus) to acclimatize for 30 min. The temperature of the glass floor was maintained at 30°C. Light from a halogen bulb lamp was delivered to the plantar surface of the mouse hindpaw through the base of the glass panel to induce the heat stimuli. The time taken for the mouse to lift or lick its hindpaw was recorded automatically by the device. The intensity of the radiant heat was adjusted to reach a basal latency of 8–10 s. A cutoff time of 20 s was used to avoid tissue damage. Three measurements were performed with intervals of 1–2 min.

Staining for intraepidermal nerve fiber density (IENFD) was performed as described previously (11, 30, 31). The mean IENFD were measured using standardized measurement protocols and compared with controls (11). IENFD was determined by the number of complete baseline crossings of nerve fibers at the dermoeipidermal junction divided by the calculated length of the epidermal surface.

Western blot analysis. DRG neurons were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.4, 1% SDS, 1% Triton X-100, and 150 mM NaCl). Proteins (25 μg) were extracted and SDS-PAGE gels prepared as described previously (12). Nitrocellulose membranes were probed with anti-mouse-specific TFAM antibody (cat. no. 28-597; Prosci), anti-human-specific TFAM antibody (cat. no. 7495; Cell Signaling Technology), or antibody recognizing both mouse and human TFAM (cat. no. SAB1401383; Sigma-Aldrich). Following application of species-specific secondary antibodies, the signal was detected using the Super Signal chemiluminescence kit (Pierce), and the signal intensity was evaluated using an Alpha Innotech imaging system.

Mitochondrial oxygen consumption. Oxygen consumption was measured using a thermostatically controlled Clark-type O2 electrode (Hanschek Instruments, Norfolk, UK), as described previously (49). State 3 respiration was measured with malate and glutamate substrates in the presence of ADP (10, 11). Approximately 2 min later state 3 was terminated, and state 4 respiration (resting) was initiated with the addition of the ATP synthase inhibitor oligomycin (1.25 μg/ml). The maximal rate of uncoupled respiration was subsequently measured by titration with 54 nM FCCP.

Quantitative real-time PCR measurement of mtDNA and other genes. Lumbar DRG were harvested from diabetic (after 16 wk of diabetes) and nondiabetic mice. RNA and DNA were isolated using a standard Invitrogen protocol. The ratio of mtDNA to nDNA was determined using a quantitative PCR method (10, 11). The cycle threshold (Ct) values of mtDNA and nDNA were used to determine the relative ratio of mtDNA to nDNA in DRG neurons. Relative ratio of NADH dehydrogenase subunit 1 (ND1; encoded on the mitochondrial genome) over lipoprotein lipase (LPL; encoded on the nuclear genome) was determined. Primers and PCR conditions were used as described previously (11). For other real-time PCR experiments, the Applied Biosystems primer assay IDs are as follows: TFAM, Mm00447485_m1; manganese superoxide dismutase (MN SOD/SOD2), Mm00449726_m1; glutathione peroxidase (GPX1), Mm00656767-g1. Individual genes were run against GAPDH or β-actin as controls.

Mitochondrial DNA copy number and mitochondrial DNA damage. Genomic DNA was isolated from the L5 DRG neurons. Mitochondrial DNA copy number was determined by PCR of two mtDNA targets, a 197-bp ND1 gene and a 199-bp CytB mtDNA fragments (10). Nuclear DNA copy number was determined by PCR for a nuclear DNA 175-bp B2M gene target and the CytB values were compared with a plasmid carrying a B2M nuclear gene fragment. The ratio of mtDNA to nDNA was calculated by 2 × (ND1 copies/20 ng DNA)/(B2M copies/20 ng DNA) and by 2 × (CytB copies/20 ng DNA)/(B2M copies/20 ng DNA). Damage to mtDNA was measured by long-range (LR) qPCR of an 8.9-kb mtDNA target (10). The amount of PCR product was quantified, and the amount was inversely related to mtDNA damage.

Adult mouse neuron culture and measurement of oxidative stress and cellular injury. DRG were collected from adult WT and TFAM Tg C57Bl/6j mice (11). DRG were placed in Leibovitz’s L-15 media and centrifuged to pellet, and 0.5 ml of papain (2 mg/ml in Hanks’ balanced solution) and 0.5 ml of collagenase (2.5% in sterile water; Worthington) were added and incubated for 30 min at 37°C. After 30 min, 2 ml of FBS (Atlanta Biological) was added to inhibit the enzymes, and cells were centrifuged, processed, and plated as described (11). The final concentration of media components was as follows: selenium (5.2 μg/ml), hydrocortisone (7.6 μg/ml), transferrin
Characterization of hTFAM transgenic mouse. hTFAM cDNA was used to generate the Tg mice and was mated with WT C57BL6 mice to generate heterozygous hTFAM-Tg mice. The Tg mice were identified from tail DNA by PCR using primers specific to hTFAM (Fig. 1A). The promoter used to drive the expression of hTFAM was a modified chicken B-actin promoter with cytomegalovirus enhancer. To ensure that the Tg mice expressed hTFAM in DRG neurons and did not affect the expression of endogenous mouse TFAM (mTFAM), RT-PCR and Western blot analysis were performed using species-specific primers and antibodies (24). The results are shown in Fig. 1, B and C. We found in DRG neurons that hTFAM mRNA and protein were expressed only in the TFAM Tg mice and not in the WT mice. Furthermore, in DRG neurons, the mTFAM mRNA and protein levels were similar in both WT and Tg mice, which demonstrated that the expression of TFAM did not affect the expression of endogenous mTFAM gene. Quantification of the intensity of the TFAM bands suggests that the total levels of TFAM (mTFAM + hTFAM) increased 80–100% in the Tg mice compared with WT mice (Fig. 1).

Expression of mtDNA and mitochondrial respiration. We tested whether the expression of hTFAM is functional in the Tg mouse, and does it regulate the levels of mouse mtDNA? The result is shown in Fig. 2. We performed Southern blot analyses to assess mtDNA levels in total DNA extracts from DRG neurons from WT and Tg animals (Fig. 2A, left), and found a clear increase in mtDNA levels in DRG neurons from the Tg animals. The overall increase in mtDNA in Tg mice compared with the WT mice was ~90% (Fig. 2A, middle), and this increase in mtDNA paralleled the increase in hTFAM protein levels. The calculated ratio between TFAM protein and mtDNA remained the same in both WT and Tg mice (Fig. 2A, right). Mitochondria were isolated from lumbar sensory DRG neurons of 6-mo-old WT and Tg mice, and mitochondrial state 3 and state 4 respiration was measured. The results are shown in Fig. 2B. ADP-stimulated state 3 and oligomycin-sensitive state 4 respiration rates were ~20% higher in Tg mice compared with the WT mice. The increase was not statistically significant. There was no significant difference in respiratory control ratio between the groups.

Characteristics of WT and TFAM Tg control and diabetic mice. Diabetes was induced in 3-mo-old WT and TFAM Tg mice by the administration of STZ. The body weight, blood glucose levels, and blood lipid levels are shown in animal groups at 6 and 16 wk (Table 1). Both WT diabetic and TFAM Tg diabetic mice lost a significant amount of weight. Fasting blood glucose was significantly elevated, and insulin was decreased in both WT diabetic and TFAM Tg diabetic mice. There was no significant difference between the diabetic groups in these values, even though TFAM transgene was expressed in all tissues. Total cholesterol was significantly increased in TFAM Tg diabetic compared with WT diabetic mice (232 ± 64.34 vs. 128.3 ± 29.6). More importantly, HDL
cholesterol, the critical cholesterol scavenger and transporter, was significantly higher in TFAM Tg diabetic mice compared with WT diabetic mice (110.5 ± 6.83 vs. 73.9 ± 7.56).

Mitochondrial degeneration in DRG neurons of WT and TFAM Tg diabetic mice. We compared the ratio of mtDNA to nDNA in DRG neurons from WT and TFAM Tg mice before and after 6 or 16 wk of STZ-induced diabetes. We used real-time PCR to obtain a relative ratio of ND1 (a gene coded by mtDNA) over LPL (a unigene encoded by nDNA). The ratio is an indicator to obtain a relative ratio of ND1 (a gene coded by mtDNA) over LPL (a unigene encoded by nDNA). The ratio is an indicator of relative mtDNA levels. The results (Fig. 3A) showed a significant decrease (40%, P < 0.05) in ND1 to LPL in DRG neurons after 16 wk of diabetes compared with nondiabetic WT mice. In TFAM Tg diabetic mice, there was a decrease (~30%) in mtDNA in in DRG neurons after 16 wk of diabetes compared with nondiabetic TFAM Tg mice. The decrease was not significant when compared with WT nondiabetic mice. Thus, compared with WT nondiabetic mice, after 16 wk of diabetes, there was no decrease in mtDNA in TFAM Tg diabetic mice. Chronic diabetes decreases mtDNA levels in DRG neurons, and the overexpression of TFAM Tg was able to retain the levels of mtDNA within the WT nondiabetic range.

Western blot analysis was done in the protein extracts of isolated mitochondria from DRG neurons of WT and TFAM Tg mice. Values were calculated from the densitometry intensity of the bands measured by Southern blot for mtDNA and Western intensity for TFAM protein. TFAM values in Tg were calculated as a ratio to WT values.

Table 1.

<table>
<thead>
<tr>
<th>Nondiabetic vs. Diabetic Animals</th>
<th>WT</th>
<th>WT Diab</th>
<th>TFAM Tg</th>
<th>TFAM Tg Diab</th>
<th>Significance (P Value)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 vs. 2</td>
</tr>
<tr>
<td>Weight, g</td>
<td>29.7 ± 0.7</td>
<td>24.4 ± 1.1</td>
<td>31.1 ± 0.5</td>
<td>23.8 ± 1.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Blood glucose, mg/dl</td>
<td>159 ± 8</td>
<td>479 ± 53</td>
<td>142 ± 15</td>
<td>459 ± 45</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Insulin, μU/ml</td>
<td>4.50 ± 0.3</td>
<td>1.87 ± 0.36</td>
<td>6.15 ± 0.12</td>
<td>1.52 ± 0.02</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>85.3 ± 5.9</td>
<td>120.3 ± 19</td>
<td>86.8 ± 1.7</td>
<td>225 ± 52.5</td>
<td>NS</td>
</tr>
<tr>
<td>HDL, mg/dl</td>
<td>59 ± 1.9</td>
<td>64.4 ± 6.33</td>
<td>58.8 ± 1.67</td>
<td>107.5 ± 7.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>58 ± 9.2</td>
<td>133.2 ± 20</td>
<td>59.33 ± 6</td>
<td>144.6 ± 7.1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>16 Wk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight, g</td>
<td>31.7 ± 0.9</td>
<td>22.4 ± 1</td>
<td>33.1 ± 0.9</td>
<td>21.8 ± 1.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Blood glucose, mg/dl</td>
<td>163 ± 12</td>
<td>467 ± 48</td>
<td>146 ± 18</td>
<td>454 ± 52</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Insulin, μU/ml</td>
<td>5.49 ± 0.3</td>
<td>1.78 ± 0.3</td>
<td>5.85 ± 0.23</td>
<td>1.63 ± 0.08</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>88.6 ± 6.98</td>
<td>128.3 ± 30</td>
<td>89.68 ± 3.7</td>
<td>232 ± 64.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>HDL, mg/dl</td>
<td>58.97 ± 3.7</td>
<td>73.9 ± 7.56</td>
<td>54.82 ± 3.5</td>
<td>110.5 ± 6.8</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>48 ± 8.7</td>
<td>126.2 ± 16</td>
<td>54.33 ± 9.5</td>
<td>102.5 ± 9.3</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Values are means ± SE. WT, wild type; Diab, diabetic; TFAM, mitochondrial transcription factor A; Tg, transgenic; NS, not significant. For 6-wk nondiabetic vs. diabetic animals, n = 6 WT, 12 WT Diab, 6 TFAM Tg, and 9 TFAM Tg Diab; for 16-wk nondiabetic vs. diabetic animals, n = 5 WT, 10 WT Diab, 8 TFAM Tg, and 8 TFAM Tg Diab.

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In addition to determining the ratio of mtDNA to nDNA, we also quantified the copy number of mtDNA. The results (Table 2) showed that there was a significant, approximately twofold increase in mtDNA copy number in DRG neurons from TFAM Tg mice compared with WT mice (TFAM Tg: 2,206 ± 320 vs. WT: 1,145 ± 175, P < 0.01). Sixteen weeks of diabetes caused a significant decrease in the mtDNA copy number in WT mice (nondiabetic: 1,145 ± 175 vs. diabetic: 630 ± 75, P < 0.01) and in TFAM Tg mice (nondiabetic: 2,206 ± 320 vs. diabetic: 1,070 ± 190, P < 0.01). The mitochondrial DNA copy number in TFAM Tg diabetic mice was not significant when compared with WT nondiabetic mice (TFAM Tg diabetic: 1,070 ± 190 vs. WT nondiabetic: 1,145 ± 175). We also determined the damage to mtDNA by LR-QPCR of an 8.9-kb mtDNA target. This was based upon the principle that DNA damage slows down or blocks the progression of DNA polymerase along a template. The results showed that there was a higher number of long-range mtDNA PCR products in TFAM Tg diabetic mice compared with WT diabetic (TFAM Tg diabetic: 965 ± 103 vs. WT diabetic: 480 ± 53). The calculated ratio of mtDNA damage to ND1 copy number showed no significant difference between WT diabetic and TFAM diabetic (WT diabetic: 64 ± 12 vs. TFAM Tg diabetic: 68 ± 18), suggesting that there is only a decrease in mtDNA copy number in chronic diabetes.

Nerve conduction and sensory testing evidence of neuropathy in TFAM Tg mice. After 16 wk of diabetes, sciatic and tail motor conduction velocities were reduced and tail sensory latencies prolonged in WT diabetic compared with WT nondiabetic mice (Table 3). In contrast, there was no significant difference in conduction velocities and latencies between TFAM Tg diabetic mice compared with nondiabetic TFAM Tg mice (Table 3). The results for von Frey sensory testing for mechanical allostynia and Hargreaves’ testing for thermal nociception after 16 wk of diabetes is shown in Fig. 4. The threshold for mechanical allostynia was lower in WT diabetic animals compared with TFAM Tg diabetic mice, consistent with more severe neuropathy in WT diabetic compared with TFAM Tg diabetic mice. The threshold for mechanical allostynia was similar in TFAM Tg diabetic mice compared with WT nondiabetic mice. Similarly diabetic mice had an increase in the thermal nociception latency after 16 wk of diabetes (Fig. 4). In the TFAM Tg mouse, the thermal nociception latency was similar to WT nondiabetic mice.

Intraepidermal nerve fiber innervation in WT diabetic and TFAM Tg diabetic mice. As shown in Fig. 5A, protein gene product (PGP) 9.5-immunoreactive nerve fibers were abundant in both the epidermis and dermis of WT and TFAM Tg nondiabetic control mice. Sixteen-week diabetic WT mice showed a significant decrease in the IENFD compared with WT nondiabetic mice and TFAM Tg diabetic mice (Fig. 5A). The mean IENFD was significantly decreased in diabetic WT mice compared with TFAM Tg diabetic mice (P < 0.001; Fig. 5B).

TFAM Tg expression prevents oxidative stress in DRG neurons. To test whether TFAM Tg expression prevents oxidative injury in neurons, adult mouse DRG neurons were prepared from 3-mo-old TFAM Tg and WT mice. Figure 6A represents an average of four separate experiments and shows that TFAM Tg expression prevents high-glucose-induced oxidative stress over 5 h using H2DCF, a marker of oxidative stress. In contrast, WT DRG neurons were highly susceptible.
to high glucose-induced oxidative stress. The bright-field and fluorescence images of the neurons are shown in Fig. 6B.

We determined whether the protection against oxidative stress by high glucose in TFAM Tg DRG neurons was due to increased expression of antioxidant enzymes. Total RNA was isolated from 3-mo-old WT and TFAM Tg mice, and the expression levels were measured by RT-PCR of scavenging enzymes as mitochondrial superoxide dismutase (SOD2) and GPX. The results are shown in Fig. 7. No significant differences in the expression of SOD2 mRNA and GPX mRNA were observed in TFAM Tg mice, suggesting that there is no increase in the capacity of the antioxidant pathway in TFAM Tg mice.

Table 2. Mitochondrial DNA copy number and DNA damage in WT and TFAM Tg mice

<table>
<thead>
<tr>
<th>Tissue</th>
<th>B2M Copies/10 ng DNA</th>
<th>ND1 Copies/10 ng DNA</th>
<th>Ratio (mtDNA/nDNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>10,600 ± 1,215</td>
<td>12,237K ± 130K</td>
<td>1,145 ± 175</td>
</tr>
<tr>
<td>TFAM Tg</td>
<td>10,250 ± 1,350</td>
<td>22,350K ± 210K</td>
<td>2,206 ± 320**</td>
</tr>
<tr>
<td>WT Diab</td>
<td>11,350 ± 1,250</td>
<td>7,160K ± 697K</td>
<td>630 ± 75</td>
</tr>
<tr>
<td>TFAM Tg Diab</td>
<td>12,720 ± 1,200</td>
<td>13,890K ± 190K</td>
<td>1,070 ± 190**</td>
</tr>
</tbody>
</table>

Values are means ± SE. **P < 0.01. ND1, NADH dehydrogenase subunit 1; mtDNA, mitochondrial DNA; nDNA, nuclear DNA. K = 1,000.

Table 3.

<table>
<thead>
<tr>
<th>Nondiabetic vs. Diabetic Animals</th>
<th>WT</th>
<th>WT Diab</th>
<th>TFAM Tg</th>
<th>TFAM Tg Diab</th>
<th>1 vs. 2</th>
<th>3 vs. 4</th>
<th>1 vs. 3</th>
<th>2 vs. 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 Wk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.05</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Sciatic NCV, m/s</td>
<td>48.5 ± 1.7</td>
<td>42.4 ± 2.3</td>
<td>48.4 ± 2.5</td>
<td>49.7 ± 2</td>
<td>&lt;0.01</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Tail SL, m/s</td>
<td>1.04 ± 0.01</td>
<td>1.11 ± 0.02</td>
<td>0.98 ± 0.02</td>
<td>0.98 ± 0.03</td>
<td>&lt;0.01</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Tail ML, ms</td>
<td>2.13 ± 0.03</td>
<td>2.35 ± 0.03</td>
<td>2.20 ± 0.04</td>
<td>2.25 ± 0.03</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>16 Wk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.01</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Sciatic NCV, m/s</td>
<td>49.9 ± 3.2</td>
<td>36.6 ± 3.2</td>
<td>49.74 ± 4.52</td>
<td>47.45 ± 1.97</td>
<td>&lt;0.01</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Tail SL, m/s</td>
<td>1.06 ± 0.02</td>
<td>1.19 ± 0.04</td>
<td>1.12 ± 0.03</td>
<td>1.05 ± 0.02</td>
<td>&lt;0.01</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Tail ML, ms</td>
<td>2.16 ± 0.04</td>
<td>2.55 ± 0.04</td>
<td>2.19 ± 0.03</td>
<td>2.65 ± 0.04</td>
<td>&lt;0.01</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Values are means ± SE. NCV, nerve conduction velocity; SCV, sensory conduction velocity; ML, motor latency. For 6-wk nondiabetic vs. diabetic animals, n = 6 WT, 12 WT Diab, 6 TFAM Tg, and 9 TFAM Tg Diab; for 16-wk nondiabetic vs. diabetic animals, n = 5 WT, 10 WT Diab, 8 TFAM Tg, and 8 TFAM Tg Diab.
to the same extent. This suggests that the mtDNA copy number per se does not affect TFAM gene expression. The proposed explanation is that the interaction between TFAM and mtDNA is dynamic and that the presence of one increases the stability of the other. This interaction is probably beneficial from a regulatory point of view because small changes in TFAM protein levels or mtDNA levels result in rapid adjustment to maintain a constant optimal ratio between TFAM and mtDNA (16). This is supported by the findings that TFAM interacts with mtDNA to wrap mtDNA to form a nucleoid structure similar to histones in the nucleosome (1, 39, 58). Calculation of the molar ratio of TFAM protein to mtDNA provides an estimate of \( \frac{1}{1000} \) molecules of TFAM per mtDNA molecule, or one TFAM molecule per 15–20 bp of mtDNA. Since mitochondria can generate ROS due to their respiratory activity, perhaps TFAM functions in multiple roles to promote mtDNA transcription, replication, and wrapping of mtDNA to protect it from attack by ROS (1, 39, 58).

The current study presents novel evidence that the expression of hTFAM protects against diabetic neuropathy. This observation is supported by the preservation of measures of neuropathy, including nerve conduction velocity, mechanical allodynia, thermal nociception, and IENFD in diabetic TFAM Tg mice. TFAM prevents slowing of nerve conduction velocity, reduces mechanical allodynia, and decreases the loss of intraepidermal nerve fibers. The blood results showed that there was no difference in glucose or insulin levels between WT diabetic and TFAM Tg diabetic mice, although hTFAM is likely to be expressed in all tissues because the promoter is not tissue or cell specific and TFAM overexpression does not reduce the severity of diabetes. The protection against peripheral nerve injury is local and independent of glycemic control.

The current study shows that there is a net loss of both mtDNA and TFAM in chronic experimental diabetes. In 6-wk diabetic DRG neurons, there is an attempt to upregulate both mtDNA and TFAM, although the increase was not significant. It could be argued that acute exposure to hyperglycemia increases TFAM levels, mtDNA, and mitochondrial biogenesis to meet the high energy demand in neurons (56). However, where there is chronic hyperglycemia with concurrent generation of ROS, the regulation might change from physiological...
to pathological and could eventually lead to a decline in mtDNA, TFAM, and mitochondrial function. mtDNA is particularly susceptible to oxidative injury, which is due in part to the following factors: 1) its location within mitochondria where the respiratory complexes I and III are potential sites for the generation of $\text{O}_2^-$ and 2) the limited repair activity against DNA damage within mitochondria (37). Under normal conditions, the toxic effects of ROS are prevented by scavenging enzymes such as SOD, GPX, and catalase as well as by other nonenzymatic antioxidants. However, when the production of ROS becomes excessive, or if the levels of antioxidant enzymes decrease, then oxidative stress might have a harmful effect on the functional and structural integrity of biological tissue. Our results showed that the DRG neurons from TFAM Tg mice are able to scavenge high glucose-induced ROS much more efficiently than the DRG neurons from WT mice. But this scavenging ability did not appear to be due an increase in the expression of the antioxidant enzymes manganese superoxide dismutase and GPX. Our results on mitochondrial respiration (Fig. 2) showed no significant increase in ADP-stimulated state 3 or resting state 4 respiration. All of these findings suggest that the protective effect of TFAM overexpression is apparent only under chronic conditions of oxidative stress. A direct proof of this would have been to show that the mitochondrial respiratory complex activities are decreased in WT diabetic DRG mitochondria but remained normal in TFAM Tg DRG mitochondria. Paucity of tissue amount required to do such experiments prevented us from this undertaking. However, results have been obtained with isolated mitochondria from other tissues (e.g., heart) using the same hTFAM transgenic mice, the original source of our TFAM Tg mouse. The results show that despite the significant increase in mtDNA copy number in the heart from TFAM Tg mice, mitochondrial respiratory complex I, complex II, complex III, and complex IV demonstrated no significant changes in enzymatic activity compared with WT heart mitochondria (24). In infarcted myocardial (MI) heart mitochondria from WT mice, the enzymatic activities of complex I, complex III, and complex IV were significantly lower than those from WT-sham. Most importantly, there was no such decrease in the enzymatic activities of complex I, complex III, or complex IV in TFAM Tg-MI mitochondria (24). These results, together with our results, suggest that the regulation of mtDNA copy number is dissociated from that of electron transport function, although protection is noted in conditions of increased oxidative stress.

In summary, our results show that TFAM overexpression prevented a decrease in mtDNA copy number in diabetic DRG neurons, helped prevent experimental diabetic neuropathy, and protected DRG neurons from oxidative stress.
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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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