Increased Prevalence of Mitochondrial DNA Deletions in Skeletal Muscle of Older Individuals With Impaired Glucose Tolerance

Possible Marker of Glycemic Stress

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To determine the relationship between mitochondrial DNA (mtDNA) mutations and age-related impaired glucose tolerance (IGT), mtDNA from skeletal muscle of 19 volunteers, ages 55–75 years, with either IGT or diabetes and 17 age- and sex-matched control subjects was analyzed using a long-extension polymerase chain reaction (PCR) combined with a quantitative PCR. We found the common 4,977-bp deletion in 84% of the IGT/diabetes group compared with only 41% in the control group ($P < 0.02$). Multiple other deletions of different sizes were identified in 13 out of 19 IGT/diabetes patients (68%) compared with 2 out of 17 control subjects (12%) ($P < 0.002$). Because of the heterogeneity and variation in the mutations identified, we propose that these mtDNA mutations were the result rather than the cause of IGT. The increase in type and frequency of mtDNA deletions in diabetes and IGT patients may be related to oxidative damage by oxygen free radicals. These may be produced in greater amounts as a result of hyperglycemia or may be more abundant because of an abnormality in the scavenging of free radicals by antioxidants. *Diabetes* 46:920–923, 1997

Human mitochondrial DNA (mtDNA) is a double-stranded 16,569-bp closed circular molecule. Over 100 diseases caused by primary defects in mitochondrial function have been described (10). The 4,977-bp deletion of sequences between base pairs 8468 and 13446 is known as the common deletion, and its frequency is increased with advancing age in human heart (4), brain (7), and skeletal muscle (11). The prevalence of diabetes also increases with age, and at least 40% of those over the age of 65 years have evidence of glucose intolerance or insulin resistance (6). We hypothesized that impaired glucose tolerance (IGT) would be associated with an increased frequency of mtDNA deletions in older individuals. If a relationship was indeed found, it would be conceivable that hyperglycemia might be a causative factor. Hyperglycemia, the primary clinical manifestation of diabetes and IGT, has been implicated in the production of oxygen free radicals, such as the superoxide anion (O_2^-) and the hydroxyl radical (OH^-) (3,15), which may in turn contribute to mtDNA damage identifiable by modern molecular biology techniques.

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IGT, impaired glucose tolerance; OGTT, oral glucose tolerance test; PCR, polymerase chain reaction; USDA, U.S. Department of Agriculture; UVM, University of Vermont.

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RESEARCH DESIGN AND METHODS

Volunteers gave written informed consent and were studied at the University of Vermont (UVM) Clinical Research Center, Burlington, Vermont, or at the U.S. Department of Agriculture (USDA) Jean Mayer Human Nutrition Research Center on Aging at Tufts University, Boston, Massachusetts. The study was approved by both the UVM and Tufts New England Medical Center committees on human research. Procedures included a medical history and physical examination, routine laboratory tests, and a 75-g oral glucose tolerance test (OGTT). Individuals matched for age and sex were grouped based on the results of the OGTT (12). Muscle biopsy samples were obtained from the vastus lateralis muscle of each volunteer using a Bergstrom needle and standard, aseptic technique.

Total muscle DNA was isolated from a 30- to 50-mg sample of muscle tissue using standard methods. Long-extension polymerase chain reaction (PCR) was performed to rapidly map mtDNA deletions (16). The reaction mixture contained 100 ng DNA, 200 μmol/l of each dNTP, 50 pmol of each primer, 1 mmol/l $MgCl_2$, and 2 U rTth DNA polymerase (Perkin-Elmer/Cetus). The PCR program was as follows: a hot start at 94°C for 4 min; 35 cycles of 94°C for 1 min, 54°C for 1.5 min, and 72°C for 5 min; and a final extension time of 10 min at 72°C. Reaction aliquots (15 μl) were electrophoresed on 2% agarose gels and visualized with ethidium bromide staining. A serial-dilution PCR was performed to quantitate the percentage of deleted mtDNA molecules compared with total mtDNA as described by Corral-Debrinski and colleagues (4,5).

Statistical analysis. Data are presented as means ± SD. Differences between groups in age and BMI, the ratio of weight to height squared, were analyzed using Student's (two-sample) *t* tests (two-tailed). Comparisons of the frequency of deletions between groups were made using χ^2 analysis. Logistic regression models were used to examine the joint effect of subject group, age, and BMI on the presence or absence of mtDNA deletions. Analysis was done using Statistica: Complete Statistical System (StatSoft, Tulsa, OK). Probabilities of ≤0.05 were considered to be statistically significant.

RESULTS

The 36 volunteers were divided into two groups: those with normal glucose tolerance (control subjects; 10 women, 7 men) and those with IGT or diabetes (11 women, 8 men). The groups did not differ significantly in age (control

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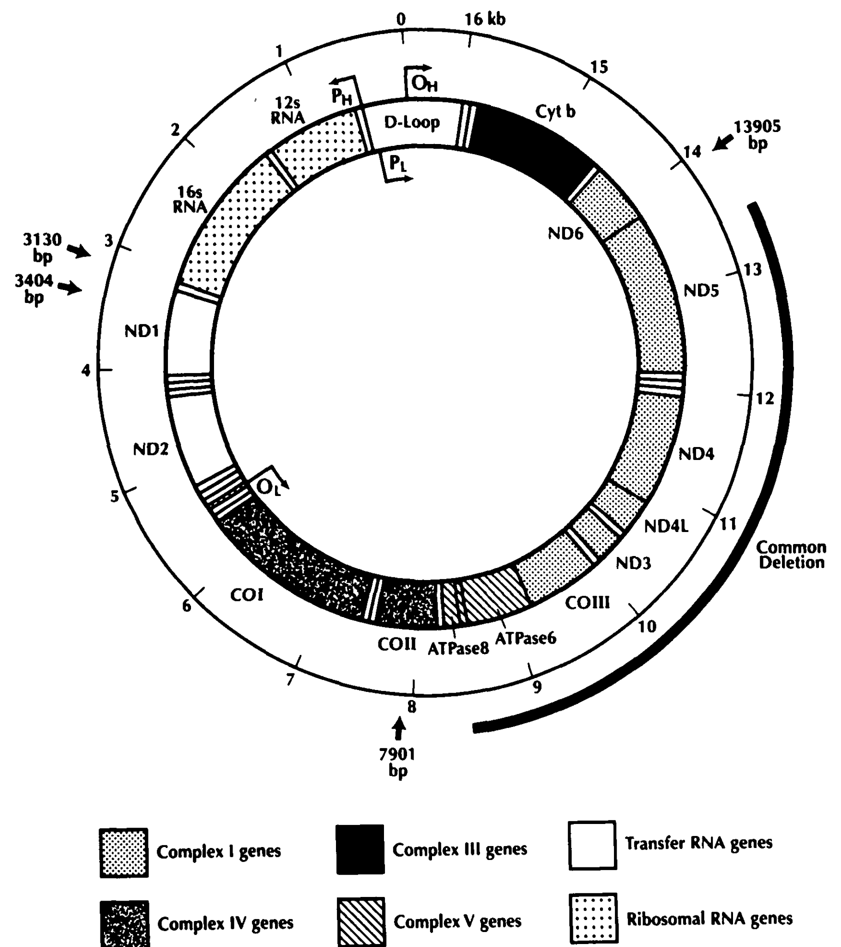

FIG. 1. Map of human mitochondrial DNA. Nucleotides 0–16569 are numbered counterclockwise from the center of the displacement (D)-loop. Shaded areas represent genes. O_H and O_L are the origins of H- and L-strand replication. P_L and P_H are the L- and H-strand promoters, respectively. The region removed by the 4,977-bp deletion is delineated by the arc outside the mtDNA circle. Arrows denote the primers selected for PCR. Oxidative phosphorylation subunits: complex I, NADH dehydrogenase (ND1, ND2, ND3, ND4); complex III, cytochrome b; complex IV, cytochrome C oxidase I, II, III; complex V, ATPase 6, ATPase 8.

group, 63 ± 5 years; IGT/diabetes group, 66 ± 6 years), BMI (29 ± 5 vs. 29 ± 4 kg/m^2, respectively), and sex distribution. Six of the IGT/diabetes subjects were considered diabetic based on the criterium of a fasting plasma glucose level >140 mg/dl or, if fasting levels were not diagnostic of diabetes, both the 2-h sample and one other during the 2-h OGTT >200 mg/dl. Three were taking oral hypoglycemic agents, and three were managed by diet alone. The other 13 had IGT as defined according to the National Diabetes Data Group criteria (12).

Use of a high-performance rTth polymerase enabled us to synthesize DNA fragments of 6,004 bp corresponding to full nondeleted sequences of mtDNA in the area of the common deletion (Fig. 1). This amplification was reproducible and without nonspecific amplification. A 1-kb fragment representing the common 4,977-bp deletion (Fig. 2*A*) was found in 16 out of 19 (84%) subjects in the IGT/diabetes group compared with 7 out of 17 (41%) subjects in the control group. The difference was significant at the $P < 0.02$ level. Logistic regression models indicated that age and BMI did not affect the differences between the two groups.

Electrophoresis of PCR products also revealed multiple bands corresponding to deletions of different sizes. The number and size of the fragments detected varied from one subject to another, but those with IGT or diabetes had increased numbers and types of mtDNA deletions (13 of 19, 68%) compared with the control subjects (2 of 17, 12%) ($P < 0.002$). These data indicate that IGT or diabetes in these older patients was associated with an increased frequency of mtDNA deletions. As with the common deletion, age and BMI did not alter the magnitude of the differences attributable to either group (i.e., glucose tolerance).

To further examine the relationship between mtDNA deletions, aging, and IGT, we calculated the ratio of total muscle mtDNA to deleted mtDNA in each of the subjects by serial-dilution PCR. The decline in PCR product for each dilution series was linear (data not shown). Figure 2*B* shows an example of the quantitative PCR of a 5,753-bp deletion. In the control group, muscle tissue from nine individuals had neither the 4,997-bp deletion nor other deletions; the rest had low levels of the 4,977-bp deletion, ranging from 0.0078 to 0.036% (mean 0.0066%) relative to wild-type mtDNA (Table 1). The individuals with IGT or diabetes had 4,977-bp deletions ranging from 0.003 to 0.78% (mean 0.0663%), demonstrating a 10-fold higher incidence of the common deletion than in control subjects ($P < 0.02$). Only 2 control subjects had mtDNA deletions distinct from the common deletion, whereas 13 subjects in the IGT/diabetes group had multiple other deletions ranging from 3,500 to 5,753 bp ($P < 0.002$). No direct relationship between the percentage of certain deletions and age was observed in our study because of a wide distribution of heterogeneous deletions.

DISCUSSION

The common 4,977-bp deletion in skeletal muscle of those with normal glucose tolerance over the age of 55 years

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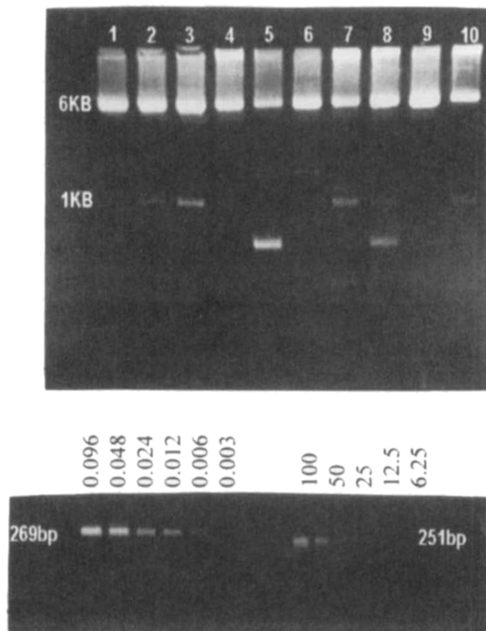

FIG. 2. *A:* agarose gel of PCR products depicting a 6-kb band representing wild-type mtDNA, a 1-kb band representing the common 4,977-bp deletion, and other bands depicting multiple different deletions. *Lane 1* is the DNA from a normal control subject. *Lanes 2–10* are DNA from patients with IGT or diabetes. *B:* serial dilution PCR of the 5,753-bp deletion. Agarose gel of PCR products representing amplification of deleted mtDNA (251 bp) and total mtDNA (269 bp). mtDNA was diluted in two ranges: one for deleted mtDNA (100–6.25 ng) and another for total undeleted mtDNA (0.096–0.003 ng). Lanes on the left show a serial dilution of the 269-bp product amplified from total DNA as the amount of template DNA is reduced from 0.096 to 0.003 ng. Similarly, as seen on the right, the 251-bp product corresponding to the 5,753-bp deletion decreases as the amount of template DNA is reduced from 100 to 6.25 ng. Consequently, the percentage of mtDNA molecules carrying this deletion is calculated to be 0.05% [i.e., 1 × 100/(6.25 ÷ 0.003)].

ranged from 0.0078 to 0.036%, consistent with many other studies (9–11). However, these data also demonstrated that the 4,977-bp deletion and several other heterogeneous deletions were significantly increased in the muscle tissue of older patients with IGT or diabetes. Although there are reports describing specific transmissible mtDNA mutations associated with diabetes (1,14), this is not the case in these individuals with IGT or diabetes. Our initial hypothesis was that the common deletion might be causally related to IGT or diabetes in the elderly. However, the heterogeneity in the mutations we found led us to conclude that IGT in older individuals was not the result of specific mtDNA mutations but rather the consequence of aging and possibly hyperglycemia. Mitochondrial DNA has been demonstrated to be more susceptible to mutations than nuclear DNA (2,13). Investigators have also shown that glucose (~25 mmol/l) and other monosaccharides have reducing properties whereby glucose can enolize and reduce molecular oxygen, yielding H_2O_2 and free-radical intermediates (15). The present findings raise the possibility that oxidative free radicals, which are continuously produced in the mitochondria by a variety of reactions under normal conditions, are produced in even greater amounts by either defects in glucose metabolism or abnormalities in the scavenging of the free radicals in the presence of hyperglycemia (8). These free radicals can then go on directly to cause mutations in mtDNA (2,13). We therefore propose that the mtDNA deletions described are markers of hyperglycemic injury rather than the initiating cause of the majority of cases of age-related IGT. Further study is needed to delineate the separate effects of age and hyperglycemia per se on mtDNA mutations. More importantly, the functional consequences of these deletions and whether therapeutic interventions aimed at reducing oxidative stress might reduce the frequency of these mutations must be examined.

TABLE 1
Summary of mtDNA deletions in patients with IGT and control subjects

	Control subjects				IGT/diabetes subjects		
Subject	Age (years)	4,977-bp deletion %	Other deletions [size (bp)]	Subject	Age (years)	4,977-bp deletion %	Other deletions [size (bp)]
HH	56	0.036	—	TJ	55	0.04	0.036 (5489), 0.018 (3500)
DG	56	0.01	—	RS	57	0.01	—
SE	56	—	—	SM	59	0.05	—
AE	57	—	—	JM	60	—	0.025 (5289)
JR	59	0.025	0.012 (5260)	EJ	63	0.003	0.001 (5509)
PJ	60	0.012	—	BJ	64	0.003	—
OM	61	—	—	HJ	64	0.012	0.012 (5509)
LA	62	0.01	—	JN	65	0.024	—
LF	63	—	0.006 (5300)	RJ	65	0.07	0.018 (5496)
CR	63	—	—	OM	67	0.01	0.05 (5509), 0.01 (5753)
DC	63	0.0078	—	MT	67	0.024	0.02 (5496), 0.018 (5753)
RJ	64	—	—	MA	67	—	0.19 (5700)
CT	65	—	—	RM	69	—	0.01 (3500)
AM	68	0.012	—	PG	69	0.01	0.04 (5289), 0.02 (5735)
CI	68	—	—	CJ	70	0.14	0.14 (5260), 0.072 (5496)
KA	71	—	—	DN	72	0.78	0.098 (5496)
RE	74	—	—	GA	74	0.024	—
				SP	75	0.01	—
				SD	75	0.05	0.05 (5753)

— indicates that no deletion was found.

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Dedicated to the memory of S. Wolff.

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