

TIME OF ONSET OF NON-INSULIN-DEPENDENT DIABETES MELLITUS AND GENETIC VARIATION IN THE β_3 -ADRENERGIC-RECEPTOR GENE

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Abstract Background. The β_3 -adrenergic receptor is expressed in visceral adipose tissue and is thought to contribute to the regulation of the resting metabolic rate and lipolysis.

Methods. To investigate whether mutations in the gene for the β_3 -adrenergic receptor predispose patients to obesity and non-insulin-dependent diabetes mellitus (NIDDM), we studied this gene in 10 Pima Indians by analysis of single-stranded conformational polymorphisms and dideoxy sequence analysis. Association studies were performed in 642 Pima subjects (390 with NIDDM and 252 without NIDDM).

Results. A missense mutation was identified in the gene for the β_3 -adrenergic receptor that results in the replacement of tryptophan by arginine (Trp64Arg) in the first intracellular loop of the receptor. This mutation was detected with allelic frequencies of 0.31 in Pima Indians,

0.13 in 62 Mexican Americans, 0.12 in 49 blacks, and 0.08 in 48 whites in the United States. Among Pimas, the frequency of the Trp64Arg mutation was similar in nondiabetic and diabetic subjects. However, in subjects homozygous for the mutation the mean (\pm SD) age at the onset of NIDDM was significantly lower (36 ± 10 years) than in Trp64Arg heterozygotes (40 ± 10 years) or normal homozygotes (41 ± 11 years; $P = 0.02$). Furthermore, subjects with the mutation tended to have a lower adjusted resting metabolic rate ($P = 0.14$ by analysis of covariance).

Conclusions. Pima subjects homozygous for the Trp64Arg β_3 -adrenergic-receptor mutation have an earlier onset of NIDDM and tend to have a lower resting metabolic rate. This mutation may accelerate the onset of NIDDM by altering the balance of energy metabolism in visceral adipose tissue. (*N Engl J Med* 1995;333:343-7.)

NON-INSULIN-DEPENDENT diabetes mellitus (NIDDM) is one of the most common inherited diseases, with an estimated prevalence of 8 to 10 percent among whites.¹ Although most forms of the disease do not have a simple mendelian pattern of inheritance, the contribution of heredity is well recognized.² It is likely that the common forms of NIDDM are complex and heterogeneous and that they result when a pool of mutant genes, each contributing in a small and subtle way, interact with one another and with environmental, aging, and behavioral influences to lead to the expression of the disease.²⁻⁴

Obesity is a known risk factor for the development of NIDDM^{5,6} and, like NIDDM, has clear genetic determinants.^{7,8} In humans, resting metabolic rate is a familial trait,^{9,10} and a low resting metabolic rate is a risk factor for weight gain and obesity.^{11,12} In rodents, resting metabolic rate is regulated by the sympathetic nervous system and acts through the modulation of lipolysis and

thermogenesis in brown adipose tissue.^{13,14} Although adult humans do not have anatomically distinct deposits of brown adipose tissue, the identification of uncoupling protein, a marker widely regarded as specific for such tissue, suggests that modulation of thermogenesis may also be important in human adipose tissue.¹⁵

The β_3 -adrenergic receptor crosses the cell membrane seven times, is coupled to guanine-nucleotide-binding (G) proteins, and is localized in adipose tissue. Stimulation of the receptor by β -adrenergic agonists activates adenylate cyclase, which increases intracellular concentrations of cyclic AMP (cAMP) and results in increased lipolysis and thermogenesis.¹⁶⁻¹⁸ There is evidence that molecular abnormalities in the β_3 -adrenergic receptor may lead to obesity and NIDDM. Expression of the receptor is markedly decreased in rodent models of obesity^{19,20}; mice with knockout (disruption) of the gene for the receptor have marked reductions in lipolysis stimulated by β -agonists,²¹ and β_3 -specific agonists have potent antiobesity and antidiabetic effects in both animals^{22,23} and humans.^{23,24} To examine further the potential role of inherited defects in this gene as contributors to obesity and NIDDM, we evaluated a group of Pima Indians, an ethnic group with a very high prevalence of these disorders, for the presence of mutations in the β_3 -adrenergic-receptor gene.

METHODS

Study Subjects

All protocols were approved by the ethics committees of the National Institute of Diabetes and Digestive and Kidney Diseases, the Indian Health Service, and the tribal council of the Gila River Indian Community and were performed after written informed consent was obtained from the subjects. The 642 subjects (390 with NIDDM and 252 without NIDDM) who participated in the main study were full-blooded Pima or Tohono O'odham Indians (or a mixture of these

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Supported by a grant (NIA 5T32AG00120) from the National Institutes of Health and by grants from the Mallinckrodt Foundation (to Dr. Shuldiner), the American Federation of Aging Research (to Dr. Shuldiner), the Chesapeake Education and Research Trust (to Drs. Walston, Silver, Austin, and Shuldiner), the Robert Wood Johnson Foundation (to Dr. Austin), and the Hartford Foundation (to Drs. Walston and Austin). Dr. Walston was supported by a Pfizer-American Geriatrics Society postdoctoral fellowship.

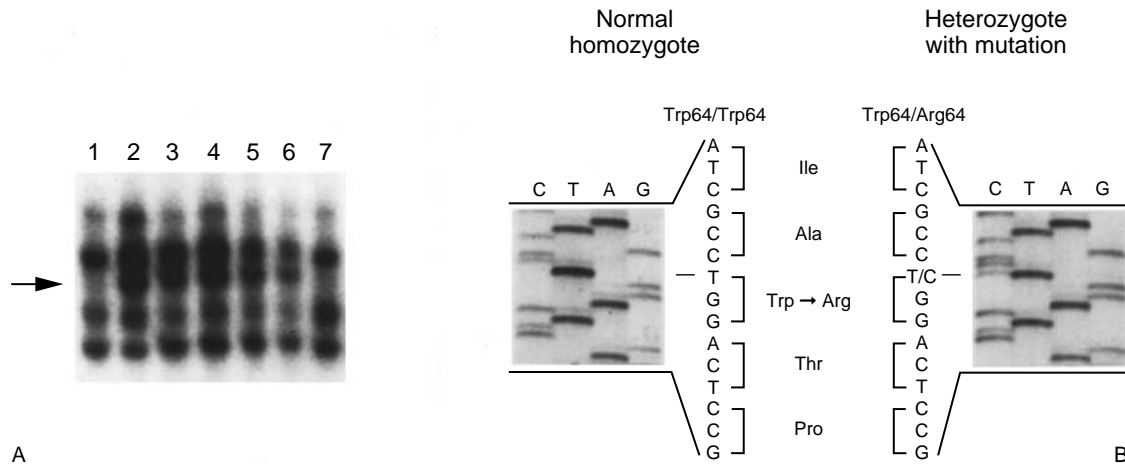


Figure 1. Identification of the Trp64Arg Mutation of the β_3 -Adrenergic-Receptor Gene.

Panel A shows an autoradiograph of an analysis of conformational polymorphisms in a single strand of DNA from a region of the β_3 -adrenergic-receptor gene in 7 of 10 diabetic Pima Indians. The arrow shows a variant pattern in lanes 2 through 6. Panel B shows autoradiographs obtained by direct sequence analysis of a region of the gene, with the normal sequence on the left. The sequencing of one of the PCR products that revealed a variant pattern in Panel A showed both a thymidine (T) and a cytosine (C) at nucleotide position 190. The patient with this pattern was therefore heterozygous for the nucleotide substitution that alters the predicted sequence of amino acids at codon 64, causing a replacement of tryptophan by arginine (TGG→CGG, or Trp64Arg).

two closely related tribes) 35 to 87 years of age. The selection of subjects was not based on their familial relationship to any other subject or to the presence of diabetes. NIDDM was diagnosed on the basis of the classification system of the World Health Organization.²⁵ Anthropometric measurements and measurements of body composition were made by standard methods.²⁶ The relations among the β_3 -adrenergic-receptor genotype, the resting metabolic rate (which was measured by indirect calorimetry with the subject in a respiratory chamber), plasma concentrations of glucose and insulin after oral glucose administration, and sensitivity to insulin (as determined by the hyperinsulinemic-euglycemic clamp technique) were studied in a separate group of 210 nondiabetic Pima Indians.^{9,26} For the estimation of allelic frequencies, β_3 -adrenergic-receptor genotypes were also determined in 62 Mexican Americans from San Antonio, Texas,²⁷ and in 49 blacks and 48 whites from the Baltimore metropolitan area.

Analysis of Single-Stranded Conformational Polymorphisms of the β_3 -Adrenergic-Receptor Gene

Genomic DNA was prepared from leukocytes or immortalized cell lines by established methods. Genomic DNA was amplified by the polymerase chain reaction (PCR) as described elsewhere.²⁸ Ten pairs of primers were used to generate 10 overlapping PCR products encompassing the entire coding region, the 5' untranslated region, the exon-intron splice junctions, and 521 base pairs (bp) of the regulatory region of the β_3 -adrenergic-receptor gene^{17,20,29} (and data deposited with the National Auxiliary Publications Service*). For the analysis of single-stranded conformational polymorphisms, the PCR products were radiolabeled by the addition of [α -³²P]deoxycytidine triphosphate to the reaction mixture. Denatured PCR products were loaded onto a polyacrylamide gel (MDE, AT Biochemicals, Malvern, Pa.), and subjected to electrophoresis at 4 to 6 W for 18 to 20 hours under four gel conditions: with and without 10 percent glycerol at

4°C and at 25°C.³⁰ The gel was vacuum-dried, and autoradiography was performed.

Dideoxy Sequence Analysis

Variants detected by analysis of single-stranded conformational polymorphisms were subjected to direct dideoxy sequence analysis with asymmetric PCR²⁸ and Sequenase Version 2.0 kits (United States Biochemicals, Cleveland). Changes in bases were confirmed by the sequencing of the opposite strands and by digestion with restriction enzymes.

Detection of the Mutated Receptor by Hybridization with Allele-Specific Oligonucleotides

A 367-bp fragment of the β_3 -adrenergic-receptor gene encompassing the mutation site was amplified by PCR with primers 5'TTCCTTCTTTCCCTACCGCCC3' and 5'GCAGCCAGTGGCGCCC-AACGG3'. The PCR products were blotted in duplicate onto nylon membranes. Hybridization was accomplished with ³²P-radiolabeled oligonucleotides corresponding to either the normal sequence of the β_3 -adrenergic-receptor gene (5'CATCGCCCTGGACTCCGA3'; the probe for Trp64) or the sequence of the Trp64Arg β_3 -adrenergic-receptor gene (5'CATCGCCCGGACTCCGA3'; the probe for Arg64).³¹ The membranes were then washed twice in 2× sodium chloride-sodium phosphate-EDTA (SSPE) (1× SSPE is 150 mM sodium chloride, 10 mM sodium phosphate, and 1.25 mM EDTA; pH 7.4) and 0.05 percent sodium dodecyl sulfate at 60°C (the Trp64 probe) or 62°C (the Arg64 probe) for 15 minutes, and autoradiography was performed.

Statistical Analysis

In the studies of the association of variables with genotype, chi-square tests were performed. In the analysis of quantitative traits, an analysis of variance was used and, where appropriate, covariates were included in the models.

RESULTS

Analysis of Single-Stranded Conformational Polymorphisms

Ten Pima Indians with obesity and NIDDM, none of whom were first-degree relatives of each other, were

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initially screened for variation in the β_3 -adrenergic-receptor gene. Analysis of single-stranded conformational polymorphisms revealed two variant patterns in the coding region. Dideoxy sequence analysis of one variant (present in 2 of the 10 Pimas) revealed a silent replacement of cytosine (C) by thymidine (T) at nucleotide position 381 (codon 127; i.e., ACC^{Thr}→ACT^{Thr}). Sequence analysis of the second variant (present in 5 of the 10 Pimas) revealed a heterozygous pattern with a replacement of thymidine (T) by cytosine (C) at nucleotide position 190 (Fig. 1). This change in bases predicted a replacement of tryptophan (TGG) by arginine (CGG) at position 64 (Trp64Arg), an amino acid in the first of the three intracellular loops of the receptor (Fig. 2). This change in bases was confirmed by restriction-enzyme digestion with *Bst*NI (Fig. 3). To screen for mutations that may have been missed in the analysis of single-stranded conformational polymorphisms, the entire coding region of the β_3 -adrenergic-receptor gene was sequenced in two additional Pima Indians with NIDDM. No new base changes were identified.

Allelic Frequencies of the Trp64Arg Mutation in Pima Indians and Other Subjects

Genomic DNA from 642 Pima Indians (390 subjects with NIDDM and 252 without NIDDM) was subjected to genotyping for the Trp64Arg missense mutation in the β_3 -adrenergic receptor by hybridization with allele-specific oligonucleotides (Fig. 4). The frequency of the Trp64Arg allele was 0.31. Nine percent of the subjects were homozygous for the mutation, 45 percent were heterozygous, and 46 percent lacked the mutation. When the genotypes were analyzed according to the subjects' age and sex, there was a statistically significant underrepresentation of Trp64Arg homozygotes among men 45 or older ($P=0.02$) (Table 1).

The frequency of the Trp64Arg allele among the 62 Mexican Americans (with 124 alleles) was 0.13. Among the 49 blacks (with 98 alleles) it was 0.12, and among the 48 whites (with 96 alleles) it was 0.08.

The Trp64Arg Genotype, NIDDM, and Obesity

Diabetes was not significantly associated with the Trp64Arg genotype in the 642 Pima Indians; the prevalence of NIDDM was 72 percent, 60 percent, and 60 percent, respectively, among Trp64Arg homozygotes, Trp64Arg heterozygotes, and normal homozygotes ($P=0.19$). From these data we concluded that the prevalence of NIDDM was slightly but not significantly higher among Trp64Arg homozygotes than among the Pima subjects with the other two genotypes (prevalence-rate ratio, 1.2; 95 percent confidence interval, 1.0 to 1.5). The mean age of the Trp64Arg homozygotes at the onset of NIDDM was significantly lower than that of the heterozygotes and the normal homozygotes ($P=0.02$) (Table 2). In view of this finding, we also examined the prevalence of diabetes that was diagnosed before the age of 25 years. Overall, this prevalence did not differ significantly between the three

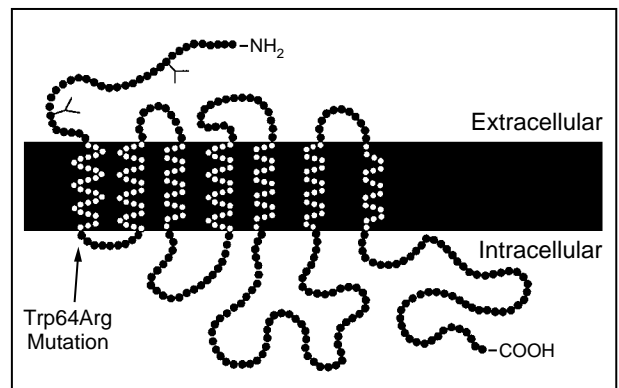


Figure 2. Diagram of the β_3 -Adrenergic Receptor. Each amino acid is shown as a circle. The Trp64Arg mutation appears at the beginning of the first intracellular loop.

groups ($P=0.11$), although our data showed that the prevalence of diabetes diagnosed before the age of 25 years was higher among Trp64Arg homozygotes than among subjects with either of the other two genotypes (prevalence-rate ratio, 2.7; 95 percent confidence interval, 1.1 to 6.8).

There was a trend toward a higher mean (\pm SD) body-mass index (the weight in kilograms divided by

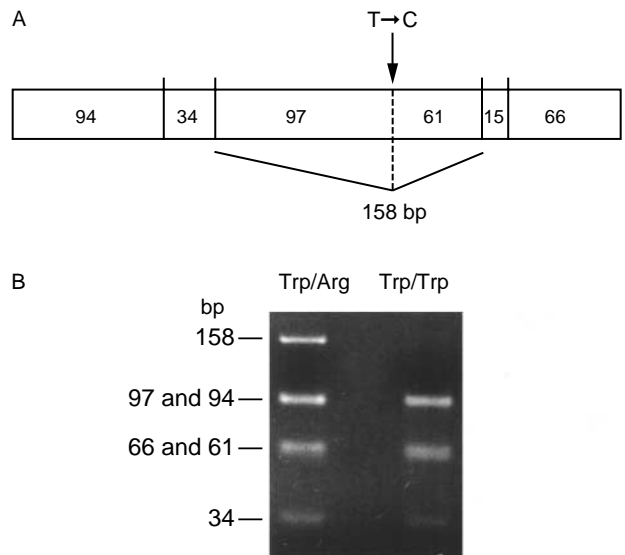


Figure 3. Use of Restriction-Enzyme Digestion to Confirm the Presence of the Trp64Arg Mutation.

Panel A shows a restriction map of the 367-bp PCR product used for digestion with *Bst*NI. Digestion of the normal sequence yields fragments of 15, 34, 61, 66, 94, and 97 bp in length, whereas the Trp64Arg mutation eliminates one of the *Bst*NI sites (dashed line), yielding a novel 158-bp product. Panel B shows an ethidium bromide-stained gel after the digestion of two DNA samples with *Bst*NI. One subject was heterozygous for the Trp64Arg mutation of the β_3 -adrenergic-receptor gene (Trp/Arg), and the other was homozygous for the normal receptor (Trp/Trp). As predicted, the mutation eradicated one of the five *Bst*NI sites in the normal sequence, causing a 158-bp product that is not normally present to appear.

the square of the height in meters) among the subjects homozygous for the Trp64Arg mutation (35.2 ± 8.0) than among subjects heterozygous for the mutation (34.1 ± 7.9) or the normal homozygotes (33.9 ± 7.5) (Table 2). Although this trend persisted after adjustment for age and sex, the differences were not statistically significant; nor were there significant differences in the ratio of the waist to the hip circumference.

The Trp64Arg Mutation and the Resting Metabolic Rate

When we studied the separate group of 210 Pima Indians, we found a trend toward a lower resting meta-

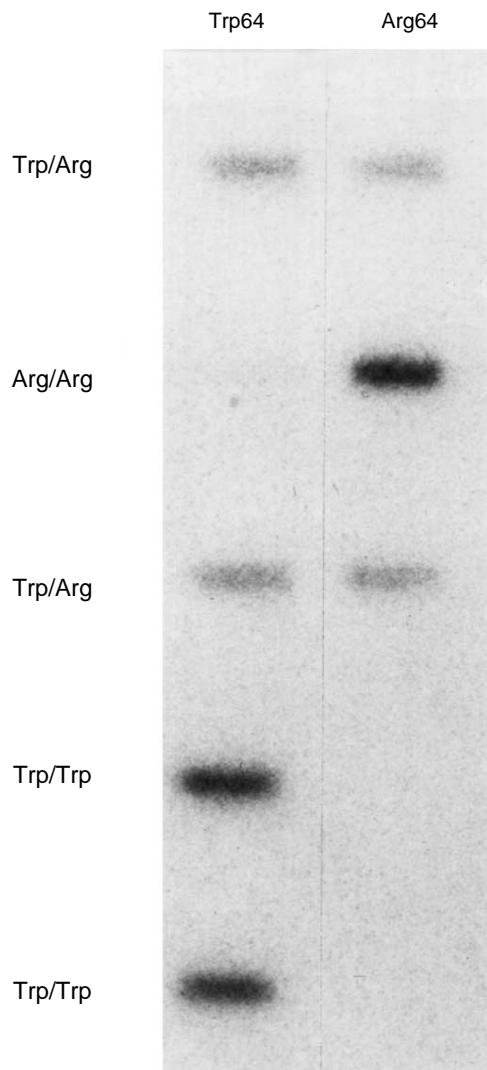


Figure 4. Detection of Mutated and Normal β_3 -Adrenergic Receptors by Hybridization with Allele-Specific Oligonucleotides. The 367-bp PCR products encompassing the Trp64Arg mutation in five Pima Indians were subjected to slot blot analysis in duplicate with oligonucleotide probes that specifically recognize either the normal β_3 -adrenergic receptor (the Trp64 probe) or the mutation (the Arg64 probe), and autoradiography was performed. Normal homozygotes (Trp/Trp), heterozygotes (Trp/Arg), and Trp64Arg homozygotes (Arg/Arg) could be detected rapidly with this assay.

Table 1. Frequency of Homozygosity for the Trp64Arg Mutation of the β_3 -Adrenergic-Receptor Gene among the Pima Indians Studied, According to Age.

AGE (YR)	MEN*	WOMEN	BOTH
<i>no. of homozygotes/no. studied (%)</i>			
35-44	10/77 (13)	17/158 (11)	27/235 (11)
≥45	7/162 (4)	23/245 (9)	30/407 (7)

*P=0.02 for the comparison between age groups.

bolic rate in subjects with the Trp64Arg mutation of the β_3 -adrenergic receptor (Table 2). After we adjusted for known covariates of the resting metabolic rate (fat-free mass, fat mass, and sex), the 22 subjects homozygous for the mutation expended an average of 82 kcal per day less than the 82 normal homozygotes; in the 106 heterozygotes, the average resting metabolic rate (36 kcal per day less than that of the normal homozygotes) was intermediate between the values for the two other groups (P=0.14 by analysis of covariance). There were no significant associations of genotype with plasma concentrations of glucose or insulin during an oral glucose-tolerance test or with sensitivity to insulin as measured by the hyperinsulinemic-euglycemic clamp technique in this group.

DISCUSSION

We have identified a mutation in the β_3 -adrenergic-receptor gene that, although not in itself associated with NIDDM, was associated with the onset of NIDDM at an earlier age among Pima Indians homozygous for the mutation. This finding suggests that the mutation is not a major determinant of NIDDM in these subjects, but rather acts to accelerate the course of the disease. The underrepresentation in the study sample of men 45 or older who were homozygous for the mutation may indicate early mortality, perhaps due to the long-term complications of diabetes that would be expected if the disease had an earlier onset.

Very small decrements in the resting metabolic rate can lead to excess accumulation of energy, weight gain, and obesity. In prospective studies of adult Pima Indians, a small daily difference in the metabolic rate (70 kcal) was a risk factor for weight gain and obesity.¹² Indeed, subjects with the Trp64Arg mutation in the gene for the β_3 -adrenergic receptor tended to have lower resting metabolic rates but were no more obese than normal subjects. Prospective measures of weight gain in younger subjects and association studies in subjects less genetically predisposed to obesity will be needed to define further the influence of this mutation on low energy expenditure and the development of obesity and NIDDM. Indeed, data from Widén and Clément and their colleagues,^{32,33} in addition to our own studies of Mexican Americans (unpublished data), implicate this mutation, even in its heterozygous form, as a contributor to central obesity and weight gain, as well as to insulin resistance and NIDDM with an accelerated on-

Table 2. Characteristics of 642 Pima Indians According to Genotype of the β_3 -Adrenergic Receptor.*

CHARACTERISTIC	Trp64Arg HOMOZYGOTES	HETEROZYGOTES	NORMAL HOMOZYGOTES
No. of subjects	57	290	295
Sex — M/F	17/40	105/185	117/178
Age — yr	48±10	51±10	51±11
Diabetes — no. (%)	41 (72)	173 (60)	176 (60)
Age at diagnosis of NIDDM — yr†	36±10	40±10	41±11
Diabetes diagnosed before age of 25 — no. (%)	5 (9)	10 (3)	9 (3)
Body-mass index‡	35.2±8.0	34.1±7.9	33.9±7.5
Difference from normal homozygotes in adjusted resting metabolic rate — kcal/day§	-82	-36	0

*Plus-minus values are means ±SD.

†P=0.02 by analysis of variance for the comparison of mean age at the onset of diabetes according to genotype. No other variables differed significantly according to genotype.

‡Calculated as the weight in kilograms divided by the square of the height in meters.

§Studied in a separate group of 210 nondiabetic subjects none of whom were first-degree relatives of each other (22 were Trp64Arg homozygotes, 106 were heterozygotes, and 82 were normal homozygotes). P=0.14 by analysis of covariance for the differences between the groups after adjustment for fat-free mass, fat mass, and sex.

set. These studies provide additional evidence that this missense mutation contributes to the genetic basis of the common forms of obesity, insulin resistance, and NIDDM.

The Trp64Arg mutation appears at the beginning of the first intracellular loop of the β_3 -adrenergic receptor (Fig. 2). On the basis of studies of the related β_2 -adrenergic receptor³⁴ and rhodopsin,³⁵ the first intracellular loop is thought to be important for the proper movement of the receptor to the cell surface and possibly also for its coupling to G proteins. Defective expression at the cell surface or impaired signaling may lead to decreased lipolysis and thermogenesis in visceral fat tissue that may contribute to central obesity, insulin resistance, and NIDDM.

We are indebted to Drs. John Burton and Philip Zieve for their support; to Drs. Reubin Andres, Terri Beaty, and Chahrazad Rafizadeh-Montrose for their advice and helpful comments; and to Mr. Keith Tanner and Ms. Amy Patterson for their assistance.

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