Original Article

Variation in the eNOS Gene Modifies the Association Between Total Energy Expenditure and Glucose Intolerance

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Endothelium-derived nitric oxide (NO) facilitates skeletal muscle glucose uptake. Energy expenditure induces the endothelial NO synthase (eNOS) gene, providing a mechanism for insulin-independent glucose disposal. The object was to test *1*) the association of genetic variation in eNOS, as assessed by haplotype-tagging single nucleotide polymorphisms (htSNPs) with type 2 diabetes, and *2*) the interaction between eNOS haplotypes and total energy expenditure on glucose intolerance. Using multivariate models, we tested associations between eNOS htSNPs and diabetes (n = 461 and 474 case and control subjects, respectively) and glucose intolerance (two cohorts of n = 706 and 738 U.K. and Spanish Caucasians, respectively), and we tested eNOS × total energy expenditure interactions on glucose intolerance. An overall association between eNOS haplotype and diabetes was observed (P = 0.004). Relative to the most common haplotype (111), two haplotypes (121 and 212) tended to increase diabetes risk (OR 1.22, 95% CI 0.96–1.55), and one (122) was associated with decreased risk (0.58, 0.39–0.86). In the cohort studies, no association was observed between haplotypes and 2-h glucose (P > 0.10). However, we observed a significant total energy expenditure–haplotype interaction (P = 0.007). Genetic variation at the eNOS locus is associated with diabetes, which may be attributable to an enhanced effect of total energy expenditure on glucose disposal in individuals with specific eNOS haplotypes. Gene-environment interactions such as this may help explain why replication of genetic association frequently fails. *Diabetes* 54:2795–2801, 2005

The ability to transport glucose from the blood into muscle cells is a key factor in the pathogenesis of type 2 diabetes. Impaired muscle glucose uptake can be caused by defective insulin signal transduction in skeletal muscles (1) and endothelial dysfunction (2,3).

Nitric oxide (NO) is an ubiquitous molecule implicated in a wide range of inter- and intracellular processes (4). NO is released from endothelial cells and myocytes during the metabolism of energy and is therefore, to varying degrees, continuously synthesized. The gene encoding the enzyme that synthesizes NO is the NO synthase (NOS) gene. Three isoforms of this gene have been identified, although only two of these, neuronal NOS and endothelial NOS (eNOS), are induced via physical activity (5–7). During skeletal muscle contraction, NO levels increase markedly from basal levels by between 50 and 200% (8). Exercise training also induces chronic NO-dependent effects on vasodilatation (9).

Two of NO's many putative roles are to facilitate the uptake of glucose into skeletal muscle and to aid its subsequent metabolism (6,10). Data in rats (11) and humans (12) demonstrate that in vivo inhibition of all NOS isoforms attenuates insulin-stimulated glucose uptake by skeletal muscle and other peripheral insulin-sensitive tissue, indicating that glucose uptake via insulin signaling pathways is NO dependent (11). The main mechanism through which energy expenditure interacts with NO to facilitate glucose uptake in muscle involves vasodilatation, capillary recruitment, and hyperemia (13). However, in animals partially lacking the eNOS gene, this mechanism is severely disrupted (14). Furthermore, other studies of partial eNOS knockout mice demonstrate that these animals are hypertensive, hyperinsulinemic, and dyslipidemic and have ~40% less insulin-stimulated glucose uptake compared with wild-type mice (15).

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CCS, Cambridgeshire Case-Control Study; eNOS, endothelial nitric oxide synthase; htSNP, haplotype-tagging single nucleotide polymorphism; MRC, Medical Research Council Ely Study; NOS, nitric oxide synthase; SNP, single nucleotide polymorphism.

TABLE 1
eNOS haplotype characteristics stratified by case status: the Cambridgeshire CCS (n = 935)

	Haplotypes						
	111	112	121	122	212	221	222
Case subjects (n = 461)							
Haplotype frequencies (%)	28.7	15.1	12.3	4.3	8.1	21.3	7.8
Age (years) (unadjusted)	63.5 ± 0.55	63.2 ± 0.61	63.7 ± 0.7	62.7 ± 1.3	63.5 ± 0.87	64 ± 0.54	64.7 ± 0.7
Sex (% F)	37.1	36.6	32.8	32.6	31.3	34.7	31.7
Height (cm)	168.5 ± 0.41	169.4 ± 0.6	169 ± 0.55	169.6 ± 1.08	168.7 ± 0.81	169.3 ± 0.43	169.6 ± 0.7
BMI (kg/m^2)	30 ± 0.4	28.9 ± 0.33	29.4 ± 0.44	28 ± 0.51	29.2 ± 0.41	30 ± 0.4	28.9 ± 0.41
Fat mass (kg)	29.3 ± 0.81	28 ± 0.81	28.6 ± 1.01	25 ± 1.23	27.4 ± 1.02	29.7 ± 0.81	27.3 ± 0.97
Fat-free mass (kg)	54.8 ± 0.44	54.8 ± 0.52	55.2 ± 0.54	55.6 ± 0.89	55.4 ± 0.79	56.2 ± 0.68	55.2 ± 0.64
Control subjects (n = 474)							
Haplotype frequencies (%)	29.2	15.6	10.0	7.4	6.7	20.2	8.0
Age (years) (unadjusted)	63.5 ± 0.52	65 ± 0.58	62.6 ± 0.72	63.9 ± 0.86	63.9 ± 0.89	63.1 ± 0.57	64 ± 0.79
Sex (% F)	34.7	35.7	37.3	31.5	32.6	36.6	34.0
Height (cm)	170.3 ± 0.46	169.9 ± 0.54	169.7 ± 0.6	169.7 ± 0.89	170.1 ± 0.61	169.7 ± 0.44	169.4 ± 0.59
BMI (kg/m^2)	27.1 ± 0.25	26.9 ± 0.3	27.8 ± 0.4	27 ± 0.45	27.8 ± 0.49	27.7 ± 0.29	27.1 ± 0.36
Fat mass (kg)	23.9 ± 0.57	23.7 ± 0.78	25.6 ± 0.96	24.3 ± 1.14	25.3 ± 1.2	25 ± 0.7	23.8 ± 0.8
Fat-free mass (kg)	54.5 ± 0.36	54.3 ± 0.49	54.2 ± 0.6	53.1 ± 0.77	55.1 ± 0.59	54.8 ± 0.42	54.2 ± 0.56

Data are age- and sex-adjusted means ± SE, unless otherwise indicated. Difference between haplotypes for all traits: $P > 0.05$.

The purpose of the current study was to investigate the relationship between eNOS gene haplotypes and diabetes and to examine whether these haplotypes modify the relationship between total energy expenditure and glucose intolerance.

RESEARCH DESIGN AND METHODS

The current study was undertaken in a diabetes case-control study of middle-aged Caucasians (the Cambridgeshire Case-Control Study [CCS]) and two middle-aged Caucasian population-based cohorts (the Medical Research Council [MRC] Ely Study in the U.K. and the Segovia Study in Spain), one of which included data on objectively measured total energy expenditure.

The Cambridgeshire CCS, U.K. The Cambridgeshire CCS (16,17) comprises 550 case subjects with type 2 diabetes and 550 individually matched control subjects, of whom DNA and phenotype data were available in 461 case and 474 control subjects. The case subjects were a random population-based sample of U.K. Caucasian men and women with type 2 diabetes aged 47–75 years from a population-based diabetes register in a geographically defined region in Cambridgeshire, U.K. Type 2 diabetes was defined as the onset of diabetes after the age of 30 years without use of insulin therapy in the 1st year after diagnosis. The control subjects, also Caucasian, were individually age-, sex-, and geographical location–matched, with one control subject for every case subject. Control subjects were not matched to case subjects by BMI. Potential control subjects that had HbA_{1c} levels >6% were excluded because this group may contain a higher proportion of individuals with previously undiagnosed diabetes. Further details on the characteristics of the subjects are shown in Table 1. Ethical permission for the study was granted by the Cambridge local research ethics committee.

MRC Ely Study, U.K. The MRC Ely Study is a prospective population-based cohort study of the etiology and pathogenesis of type 2 diabetes and related metabolic disorders. The sample selection procedures have been described in detail previously (18–20). Participants, all Caucasian, underwent standard anthropometric and clinical measurements, which included the quantification of fat mass and fat-free mass via bioimpedance (Bodystat, Isle of Man, U.K.) and a 75-g oral glucose tolerance test. Free-living total energy expenditure was assessed objectively in each participant using the flex heart rate technique. This method has been shown to be a reliable and valid method of assessing energy expenditure when compared with the gold standard methods of doubly labeled water and indirect calorimetry (18–20). Briefly, flex heart rate involves the individual calibration of heart rate against energy expenditure assessed using indirect calorimetry at rest and during an exercise stress test. All methods for this study have been described in detail previously (21,22). In the current analyses, total energy expenditure is defined as total free-living energy expenditure, and the statistical analyses are adjusted for age, sex, fat mass, and fat-free mass. Because fat mass and fat-free mass are the main determinants of resting energy expenditure, the residual variance in total energy expenditure is largely reflective of the level of habitual physical activity energy expenditure. For the current analyses, data were available in 706 healthy nondiabetic participants (age 53.4 ± 10.7 years [means ± SD], male n = 309, female n = 397). Further details on the characteristics of the subjects are shown in Table 2. Ethical permission for the study was granted by the Cambridge local research ethics committee.

The Segovia Study, Spain. The Segovia Study was designed as a cross-sectional population-based study of the prevalence of anthropometric and physiological parameters related to obesity and other components of the metabolic syndrome. It was conducted in rural and urban areas of the province of Segovia (Community of Castilla-León) in Central Spain. The methods for this study have been described in detail previously (23). However, in brief, a random sample of 2,992 men and nonpregnant women aged 35–74 years were selected from a targeted population of 63,417 inhabitants, of which 1,033 subjects agreed to participate. Subjects with a previous diagnosis of type 1 diabetes (n = 133) were excluded from the sample. All study subjects gave written informed consent to participate in the study. The study protocol was approved by the ethics committee of the Hospital Clínico San Carlos of Madrid. A random sample (n = 738), similar in number to the MRC Ely Study, were successfully genotyped for the eNOS variants. Further details on the characteristics of the subjects are shown in Table 1.

Genetic analyses. Figure 1 shows the genomic location and the magnitude of association with diabetes (odds ratio [OR] and 95% CI) for eNOS single nucleotide polymorphisms (SNPs). Figure 2 shows the pairwise linkage disequilibrium between SNPs. Each of the study groups was genotyped for the 12 eNOS gene variants shown in Fig. 1. Five of these SNPs (IVS2+42, IVS6-26, IVS11-30, E298D –rs1799983, and IVS25+15) were identified from de novo polymorphism screening in a human diversity panel with 47 samples from four distinct ethnic groups. The remaining seven candidate SNPs (*rs31800783*, *rs31800779*, *rs32070744*, *rs33800787*, *rs3918166*, *rs3918212*, and *rs3918220*) were selected from the dbSNP database to increase coverage of the gene. The putative SNPs *rs3918166*, *rs3918212*, and *rs3918220* were monomorphic in the populations studied here. dbSNPs were genotyped at the Wellcome Trust Sanger Institute, Cambridge, U.K., using an adaptation of the homogenous MassExtend protocol supplied by Sequenom for the MassArray system. The remaining SNPs were genotyped at Incyte Genomics, Cambridge, U.K., using previously described methods (24–26). In summary, DNA from white blood cells was genotyped for each polymorphism using an adaptation of the fluorescence polarization template-directed incorporation method described elsewhere (24). Primer extension preamplification–amplified DNA samples were PCR amplified in 8-μl reactions with primers flanking the variant site, and unincorporated dNTP and remaining unused primer were degraded by exonuclease I and shrimp alkaline phosphatase at 37°C for 45 min before the enzymes were heat inactivated at 95°C for 15 min. At the end of the reaction, the samples were held at 4°C. Single-base primer extension reactions were performed as previously described (25), and allele detection was performed by measuring fluorescence polarization on an LJL Analyst fluorescent reader (Molecular Devices) (24–26). All genotypes fulfilled Hardy-Weinberg expectations ($P > 0.05$).

Statistical analysis. Analyses were conducted using either the SAS system for Windows (version 8.02; SAS Institute, Cary, NC) or Stata SE 8.2 for

TABLE 2
eNOS haplotype characteristics: the MRC Ely Study (n = 706)

	Haplotypes						
	111	112	121	122	212	221	222
Haplotype frequencies (%)	29.4	13.3	10.2	7.6	7.1	21.7	8.4
Age (years) (unadjusted)	53.6 ± 0.63	53.2 ± 0.74	52.6 ± 0.8	53.3 ± 1.06	52.6 ± 1.14	54 ± 0.68	54.1 ± 0.83
Sex (% F)	58	61	59	60	57	59	60
Height (cm)	167.6 ± 0.34	167.1 ± 0.45	167.6 ± 0.48	167.2 ± 0.5	167.2 ± 0.57	167.2 ± 0.39	167.9 ± 0.52
BMI (kg/m^2)	26.2 ± 0.25	25.9 ± 0.25	26.7 ± 0.39	26.6 ± 0.44	25.8 ± 0.38	26.8 ± 0.28	26.6 ± 0.35
Fat mass (kg)	23 ± 0.46	22.2 ± 0.44	23.8 ± 0.73	23.7 ± 0.88	22.5 ± 0.69	24 ± 0.52	23.9 ± 0.64
Fat-free mass (kg)	50.7 ± 0.34	50.2 ± 0.48	51.2 ± 0.48	51 ± 0.55	49.8 ± 0.58	51 ± 0.39	51.3 ± 0.44
Fasting glucose (mmol/l)	4.85 (4.80–4.91)	4.86 (4.79–4.93)	4.9 (4.82–4.98)	4.86 (4.77–4.94)	4.82 (4.71–4.93)	4.9 (4.80–5.01)	4.85 (4.77–4.93)
2-h glucose (mmol/l)	5.33 (5.17–5.50)	5.26 (5.05–5.48)	5.38 (5.12–5.66)	5.28 (5.03–5.55)	5.28 (5.01–5.56)	5.62 (5.39–5.87)	5.59 (5.32–5.88)
REE (kJ/min)	5.76 ± 0.06	5.71 ± 0.07	6.07 ± 0.17	5.89 ± 0.09	5.96 ± 0.13	5.92 ± 0.07	5.89 ± 0.10
PAEE (kJ/min)	11.3 ± 0.17	11.2 ± 0.24	11.1 ± 0.26	11.2 ± 0.25	11.3 ± 0.33	11.1 ± 0.19	11.1 ± 0.24

Data are age- and sex-adjusted means ± SE or geometric means (95% CI), unless otherwise indicated. $P > 0.05$ for difference between haplotypes for all traits. REE, resting energy expenditure; PAEE, physical activity energy expenditure.

Windows (StataCorp, College Station, TX). All analyses were initially conducted stratified by sex and assuming an additive genetic effect because this is the most conservative model. The results of these analyses were similar for men and women (data not shown). Therefore, subsequent analyses were undertaken using sex-combined data to preserve statistical power. Glucose data were skewed and were normalized by logarithmic transformation. The central tendency of skewed data are shown as the geometric mean (95% CI). The relationship between total energy expenditure and 2-h glucose was assessed in the MRC Ely Study cohort using multivariate linear regression modeling (PROC GLM) adjusted for age, sex, fat mass, and fat-free mass.

Haplotype-tagging SNPs (htSNPs) were selected using the htSNP program (available online at http://www-gene.cimr.cam.ac.uk/clayton/software/stata/) (27). This program operates on a Stata platform. htSNPs are the SNPs from a group of linked genetic variants that describe a large proportion of the total variance in all of the available SNPs. Three htSNPs (*rs2070744*, *IVS11-30*, and *rs3800787*) were selected that explained ~54% of the variance incumbent in all SNPs (Figs. 1 and 2). We then used the SNPHAP program to estimate the haplotype frequencies of the three htSNPs (htSNP and SNPHAP programs are available online from http://www-gene.cimr.cam.ac.uk/clayton/software).

The expectation maximization algorithm (28) is commonly incorporated into haplotype estimation programs such as SNPHAP that seek to reconstruct haplotypes where the phase of the alleles within individuals is unknown owing to a lack of parental information. We chose to use SNPHAP because it is a highly robust computational method for haplotype reconstruction (29,30). In brief, based on the allele frequencies, SNPHAP initially estimates the probability that the haplotype assignment is correct. The sum of the probability estimates for all possible haplotypes equals 1.0 within individuals, with each individual counting twice, once for each chromosome. In the case of individuals who carry heterozygous genotypes, where haplotype assignment is uncertain (i.e., more than one haplotype assignment is possible), the probability estimate is used to weight the contribution of the individual to that haplotype. Thus, an individual can contribute to more than one haplotype, and the extent of this contribution is determined by the haplotype assignment probability estimate. We implemented the SNPHAP program using 11 iterations and a minimum posterior probability of 0.001 for each inferred haplotype per iteration.

Haplotypes were reconstructed from the three htSNPs (rs2070744, IVS11-30, and rs3800787) identified in the control group of the Cambridgeshire CCS (Fig. 1), and seven common haplotypes (prevalent at >5%) were retained for analyses. The coding of the haplotypes refers to the allele at each genetic locus (i.e., 1 or 2). Each number within the haplotype is ordered according to its genomic location. Thus, the first number refers to allele 1 or 2 at the rs2070744 SNP, the second number refers allele 1 or 2 at the IVS11-30 SNP, and the third number refers to allele 1 or 2 at the rs3800787 SNP. Haplotypes are arranged in frequency order, such that haplotype 111 is the most frequent, and haplotype 222 is the least frequent. The number of participants reported for each study represents those in whom all three htSNPs were successfully genotyped. Comparison of haplotype frequencies among the U.K. cohort, the Spanish cohort, and the control group of the case-control study was performed using χ^2 tests. Haplotypes prevalent at >5% frequency were retained for further analysis.

The tests for main haplotype effects and for the total energy expenditure–haplotype interaction effects were performed using Stata regression command xi:regres weighted by the haplotype assignment probability and clustered by the individual identification to obtain robust standard errors. The main haplotypic models are adjusted for age and sex. The tests of association between total energy expenditure and 2-h glucose and the interaction analyses were adjusted for fat mass, fat-free mass, age, and sex.

RESULTS

Haplotype characteristics stratified by study cohort are shown in Tables 1–3. In brief, the two population-based

TABLE 3
eNOS haplotypes characteristics: the Segovia study (n = 738)

	Haplotypes						
	111	112	121	122	211	212	222
Haplotype frequencies (%)	8.3	5.1	26.8	12.5	27.0	11.6	6.4
Age (years) (unadjusted)	54.2 ± 1.08	52.7 ± 1.13	53.8 ± 0.68	53.1 ± 0.84	53.3 ± 0.64	54.1 ± 0.83	53.3 ± 1.2
Sex (% F)	56	59	57	58	52	51	57
Height (cm)	161.6 ± 0.5	162.4 ± 0.65	162 ± 0.32	162.3 ± 0.45	161.3 ± 0.33	161.9 ± 0.5	160.7 ± 0.58
BMI (kg/m^2)	27.3 ± 0.28	27.3 ± 0.34	27.6 ± 0.24	27.5 ± 0.27	27.4 ± 0.22	27.1 ± 0.29	27 ± 0.35
Fasting glucose (mmol/l)	4.59 (4.47–4.72)	4.7 (4.59–4.82)	4.62 (4.54–4.7)	4.81 (4.71–4.91)	4.65 (4.57–4.73)	4.79 (4.68–4.91)	4.75 (4.63–4.87)
2-h glucose (mmol/l)	5.61 (5.36–5.88)	5.61 (5.3–5.94)	5.61 (5.42–5.8)	5.8 (5.56–6.05)	5.75 (5.56–5.96)	5.82 (5.53–6.12)	6.09 (5.74–6.46)

Data are age- and sex-adjusted means ± SE or geometric means (95% CI), unless otherwise indicated. $P > 0.05$ for difference between haplotypes for all traits.

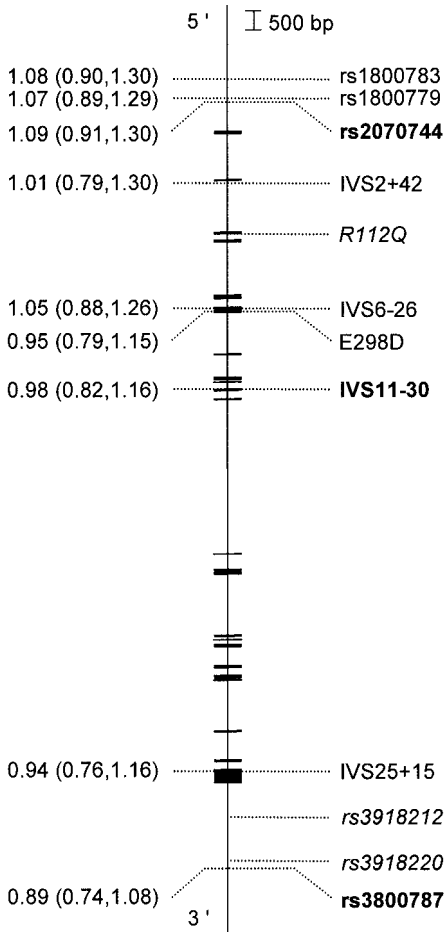

FIG. 1. Genomic location and additive risk of diabetes (on left side: OR and 95% CI) for each eNOS SNP identified in this study. Bold text denotes htSNPs. Italicized text denotes the putative SNPs that were monomorphic in this study.

control cohorts, one from the Segovia region of Spain and the other from Cambridgeshire in the U.K., comprised on average middle-aged overweight Caucasian men and women (~50% female) with normal glucose control. The Cambridgeshire CCS study comprised overweight late-middle-aged men and women (~35% female) from Cambridgeshire in the U.K., with (case subjects) and without (control subjects) type 2 diabetes.

Genetic analyses. There was no difference in haplotype frequencies between the MRC Ely Study cohort and the control cohort of the case-control study ($P = 0.80$). However, the haplotype frequencies in the Segovia cohort differed significantly from both the MRC Ely Study and the Cambridgeshire CCS control cohorts ($P < 0.0001$) (Tables 1–3).

Haplotype association with type 2 diabetes. In the Cambridgeshire CCS, several of the eNOS haplotypes were associated in the same direction and to a similar magnitude with diabetes and were thus combined into three distinct haplotype groups. These groups represented the haplotypes that, relative to the most frequent haplotype (111), showed tentative evidence for association with either an increased or decreased OR of diabetes (i.e., $P < 0.1$). For example, a tendency for increased odds of diabetes was observed in group 2 (haplotypes 121 and 212) compared with group 1 (haplotypes 111, 112, 221, and 222; OR 1.22; 95% CI 0.96–1.55) and with a decreased risk of diabetes in group 3 (haplotype 122) compared with group 1 (group 3: 0.58; 0.39–0.86). When the associations of all haplotype groups with diabetes were tested simultaneously, this model was statistically significant ($P = 0.004$ for overall difference between haplotype groups) (Fig. 3).

Haplotype association with 2-h glucose. We progressed by testing the association between the haplotype groups and glucose intolerance in the U.K. and Spanish Caucasian cohorts. Although a trend for association was observed between haplotype groups and 2-h glucose in the U.K. cohort (haplotype group 1: mean 2-h glucose 5.45 mmol/l, 95% CI 5.31–5.58; haplotype group 2: 5.35 mmol/l, 5.16–5.54; haplotype group 3: 5.27 mmol/l, 5.03–5.52), this was not statistically significant (model $P = 0.28$). In the Spanish cohort, no trend for association between haplotype groups and 2-h glucose was observed (haplotype group 1: 5.76 mmol/l, 5.57–5.96; haplotype group 2: 5.67 mmol/l, 5.51–5.84; haplotype group 3: 5.81 mmol/l, 5.07–6.06; $P = 0.51$).

Total energy expenditure × haplotype interaction analyses (MRC Ely Study only). Based on existing biological evidence, we hypothesized that the association of the level of total energy expenditure with glucose intolerance might be modified by genetic variation at the eNOS gene. In the cohort of U.K. Caucasians, objectively

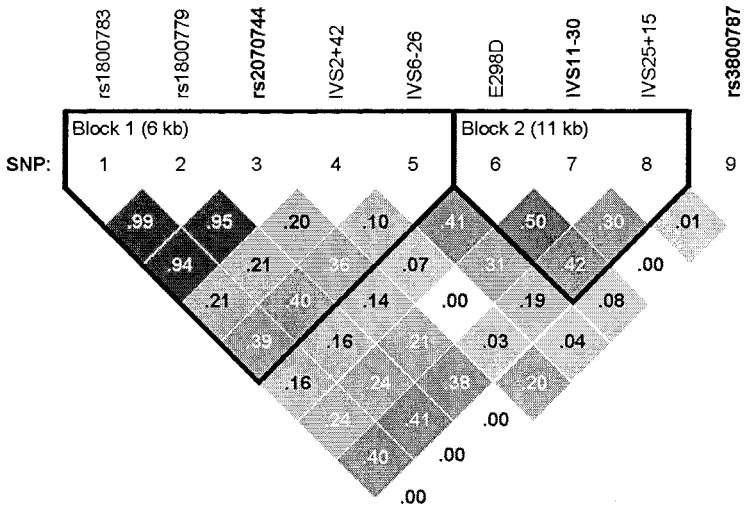

FIG. 2. Pairwise LD comparisons (r^2 shown within squares) for the nine polymorphic eNOS SNPs identified in this study. Bold text denotes htSNPs.

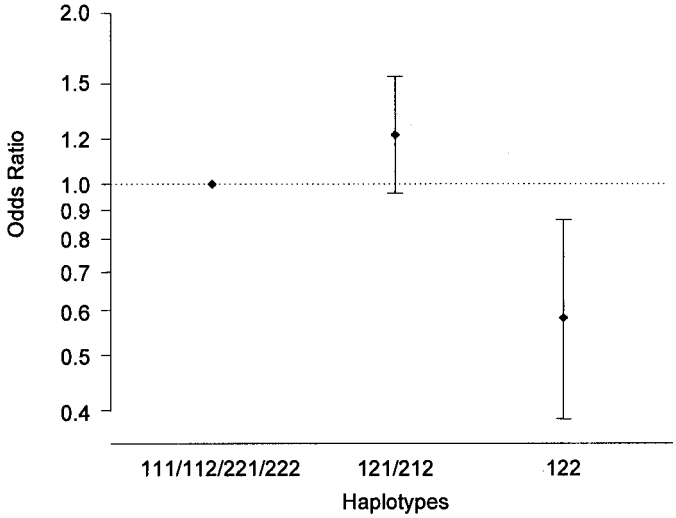

FIG. 3. Association (OR and 95% CI) between eNOS haplotype groups and risk of type 2 diabetes. The most prevalent haplotype (111) is used as the reference category where the risk estimate is 1. Statistical significance of difference in diabetes risk between haplotype groups: $P = 0.004$.

assessed total energy expenditure was strongly inversely associated with 2-h glucose (β −0.079 mmol/l per kJ energy expenditure, $P < 0.0001$). To test the hypothesis of interaction, we modeled the interaction of total energy expenditure × eNOS haplotypes, and we observed a statistically significant interaction on 2-h glucose ($P = 0.007$), indicating that eNOS gene variants modify the relationship between total energy expenditure and glucose intolerance. These models were repeated using the eNOS haplotype groups as defined above. Although total energy expenditure was inversely related with glucose intolerance in all haplotype groups (group 1: β −0.068 mmol/l per kJ energy expenditure, $P < 0.0001$; and group 2: β −0.092 mmol/l per kJ energy expenditure, $P < 0.0001$), the association between total energy expenditure and 2-h glucose was statistically stronger (total energy expenditure × haplotype group interaction $P = 0.021$) in group 3 (β −0.162 mmol/l per kJ energy expenditure, $P < 0.0001$) (Fig. 4). Although all data were analyzed in their continuous form wherever possible, data in Fig. 4 are stratified by low, moderate, and high levels of total energy expenditure.

DISCUSSION

The results of the current study indicate that variation at the eNOS gene modifies the association between total energy expenditure and glucose intolerance. This is biologically plausible because muscle contraction increases the expression of eNOS mRNA, and the molecule synthesized by the eNOS protein (NO) facilitates glucose uptake into skeletal muscle via capillary recruitment, hyperemia, and GLUT4 sequestration (7,9,10).

In the case-control study, variation at the eNOS gene was associated with risk of diabetes. However, the haplotype association is data derived and needs to be tested in independent case-control studies. We report it here to promote examination of this association in other populations. Given our primary hypothesis related to the interaction of eNOS gene variants and total energy expenditure, we would, ideally, have examined whether the association between activity and diabetes is modified by eNOS haplotypes. However, this would need to be undertaken in a case-control study nested within a large population-based cohort, and, to date, no appropriate study exists in which to do this. Although cross-sectional case-control studies exist in which physical activity has been measured through retrospective recall, these are inappropriate for testing interaction because of the issue of recall bias. In the absence of a nested case-control study, we proceeded to examine the interaction within the context of a cross-sectional cohort study in which physical activity was measured objectively and a continuous measure of glucose tolerance was available, which is not subject to recall bias in the same way as the diagnostic label of diabetes. As a prelude to examining interaction, we tested the association of eNOS haplotype with 2-h glucose as a measure of glucose tolerance. Overall, there was no statistically significant association, but in the U.K. cohort we observed a trend in the hypothesized direction, where the level of glucose intolerance observed in haplotype 122 was lower than in the reference haplotype group. The absence of an association with 2-h glucose could be a false-negative finding because of limited statistical power, with the heterogeneity of association between the two studies being attributable to population stratification at the eNOS locus. It is also possible that a true association is obscured by biological interaction between total energy expenditure and the eNOS gene. In support of this hypothesis, we observed a significantly stronger relationship between

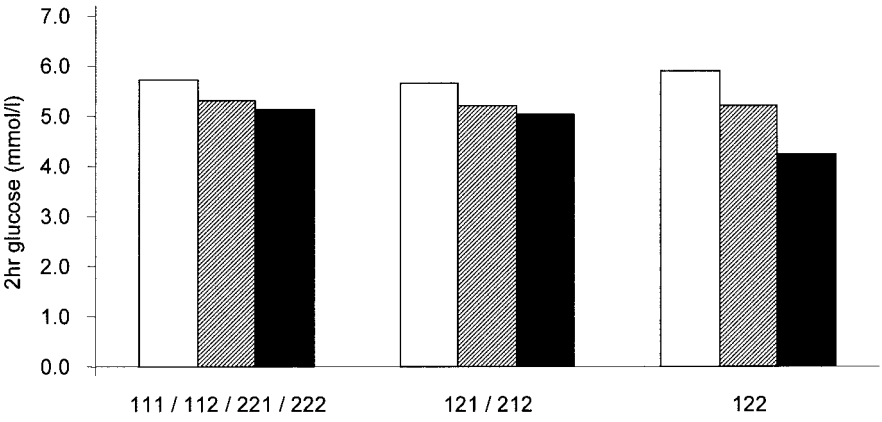

FIG. 4. Interaction between eNOS haplotype groups and total energy expenditure (TEE) on glucose intolerance. Data are stratified by tertiles of TEE: □, low levels of TEE; ▨, moderate levels of TEE; ■, high levels of TEE.

total energy expenditure and glucose tolerance in the haplotype group that, by comparison with the remaining haplotype groups, we had earlier shown to be protective of diabetes. This observation suggests that the between-individual variability in improved glucose control after physical activity intervention observed previously (31,32) may be partly attributable to genetic variation at the eNOS locus.

Evidence to support a functional role for the eNOS htSNPs in altered gene expression is lacking, although one SNP that we identified through sequencing (E298D) is nonsynonymous. Thus, we hypothesize that even if these htSNPs do not directly influence expression, they may be in linkage disequilibrium with variants that do. We therefore reconstructed haplotypes using these htSNPs and derived groups of haplotypes based on association with diabetes. The purpose of undertaking this specific approach is partly to reduce genetic information to a manageable level given the sample sizes available. Although one could theoretically test the effects of all available SNPs and haplotypes, this would either require testing a very large number of individual hypotheses (which would increase the probability of false-positive associations) or the inclusion of all SNPs or haplotypes in a single model (which would result in multiple degrees of freedom and thus, to be sufficiently powered, would require a sample size many times larger than currently available). Therefore, we opted for an approach that balances the need to adequately characterize genetic architecture with the need to preserve statistical power.

A recent meta-analysis of published studies reported evidence of linkage for fasting glucose and insulin resistance to 7q36, the genomic region to which the eNOS gene maps (33). Although large population-based cohort studies examining the relationship between variation at the eNOS gene and glucose intolerance are scarce (34), several clinical studies in people with type 2 diabetes have examined the association of eNOS polymorphisms with hypertension (35), nephropathy (36,37), retinopathy (38), and ischemic heart disease (39). Most of these studies have tested the association of the Glu298Asp (E298D) polymorphism, which is associated with basal NO production (40) and decreased flow-mediated dilatation (39). These observations may be attributable to a direct effect of E298D on mRNA levels, although this is unlikely (41), or because E298D is in high linkage disequilibrium with an unknown functional variant(s). In our data, other SNPs explain a greater proportion of the variance in 2-h glucose than E298D, indicating that the latter of these explanations may be correct.

Numerous intervention studies have reported a positive effect of physical activity on insulin sensitivity and glucose tolerance even in individuals who are insulin deficient (31,32,42–44). A possible mechanism for exercise-induced non–insulin-dependent glucose uptake involves the vasodilatory actions of NO (12,45,46). The synthesis of endothelium-derived NO increases after muscle contraction and shear stress, both of which occur during physical activity. Although in vivo and in vitro data (12,45–48) support the possibility that the eNOS gene modifies the protective effects of physical activity on vasodilatation and glucose disposal, epidemiological evidence for this hypothesis is limited. However, two studies have examined the relationship between the eNOS polymorphism and blood pressure. The first, from the HERITAGE (Health, Risk Factors, Exercise Training, and Genetics) study (49), reported association between E298D and exercising blood pressure after 20 weeks of exercise training, where blood pressure decreased most in the Glu298 allele homozygotes. In the second study, of Japanese adults (50), an interaction was described between self-report physical activity and an eNOS variant located in intron 4 on resting blood pressure. The association between physical activity and blood pressure was inverse in minor allele carriers, but it was absent in common allele homozygotes.

In summary, we have conducted a comprehensive epidemiological study of the relationship between variation at the eNOS gene locus and glucose intolerance. Our findings suggest, but do not confirm, that variation at the eNOS gene influences risk of diabetes and level of glucose intolerance and that total energy expenditure and variation within the eNOS gene interact to modify these phenotypes. If replicated and supported by other forms of evidence, such as differential response to an activity intervention by genotype, then such information could be useful in identifying individuals who are at high risk of diabetes when sedentary, but who are likely to respond well to activity interventions.

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