Variants in *ASK1* Are Associated With Skeletal Muscle *ASK1* Expression, In Vivo Insulin Resistance, and Type 2 Diabetes in Pima Indians

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OBJECTIVE—Prior genome-wide association and exon array expression studies both provided suggestive evidence that apoptosis signal regulating kinase 1 (ASK1) may influence in vivo insulin action in Pima Indians. Genetic variants in or near *ASK1* were analyzed to assess the role of this gene in insulin action and type 2 diabetes.

RESEARCH DESIGN AND METHODS—Genotypic data from 31 variants were used to determine the linkage disequilibrium pattern across *ASK1* in Pima Indians. Eight tag SNPs were initially genotyped in 3,501 full-heritage Pima Indians. Replication for association with diabetes was assessed in a second population-based sample of 3,723 Native Americans and the published DIAGRAM study. Quantitative traits were analyzed in 536 nondiabetic Native Americans, and *ASK1* expression was examined in skeletal muscle of 153 nondiabetic Native Americans.

RESULTS—Three tag SNPs were associated with type 2 diabetes (rs35898099, $P = 0.003$, odds ratio [95% CI] 1.27 [1.08–1.47]; rs1570056, $P = 0.007$, 1.19 [1.05–1.36]; rs7775356, $P = 0.04$, 1.14 [1.01–1.28]) in the full-heritage Pima Indians. The association with rs35898099 was replicated in a second sample of Native Americans ($P = 0.04$, 1.22 [1.01–1.47]), while that for rs1570056 was replicated in the DIAGRAM study of Caucasians (Z statistic based $P = 0.026$; fixed-effect model, 1.06 [1.00–1.12]). The diabetes risk allele for rs1570056 was associated with reduced insulin action as assessed by either HOMA-IR in 2,549 nondiabetic full-heritage Pima Indians ($P = 0.027$) or a hyperinsulinemic-euglycemic clamp among 536 nondiabetic Native Americans ($P = 0.02$). Real-time PCR identified a positive correlation between *ASK1* expression in skeletal muscle biopsies and in vivo insulin action ($P = 0.02$, $r = 0.23$), and the risk allele for rs1570056 was associated with lower *ASK1* expression ($P = 0.003$, $r = -0.22$).

CONCLUSIONS—*ASK1* variants may increase susceptibility to type 2 diabetes by decreasing insulin sensitivity via reduced *ASK1* expression. ***Diabetes*** **59:1276–1282, 2010**

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The Pima Indians of Arizona have an extremely high prevalence of type 2 diabetes (1). Their diabetes is characterized by obesity, decreased insulin action (insulin resistance), impaired insulin secretion, as well as increased endogenous glucose production (2), and the first three characteristics are predictive of type 2 diabetes and heritable in this population (3,4). To identify genetic variants that affect the metabolic risk factors for diabetes, we previously completed a genome-wide association study (GWAS) in 536 metabolically phenotyped nondiabetic Native Americans using the Affymetrix 100K Mapping Array (Bogardus et al., unpublished data) and also examined gene expression profiles in skeletal muscle biopsies from nondiabetic Native Americans using an Affymetrix GeneChip Exon 1.0 ST Array (Bogardus et al., unpublished data). The three traits that predict type 2 diabetes, namely obesity, measures of in vivo insulin action, and acute insulin secretion, were investigated for association in these two preliminary studies. Genes associated with the same metabolic trait in both of the two genome-wide studies (<20 genes for each of the three traits) were prioritized for further investigation. One such prioritized gene is apoptosis signal regulating kinase 1 (*ASK1*). Preliminary evidence from the GWAS suggested that one intronic single nucleotide polymorphism (SNP) in *ASK1* (rs10484491) was associated with in vivo insulin action, as determined by the hyperinsulinemic-euglycemic clamp, and evidence from the exon array study suggested that *ASK1* expression levels were also correlated with in vivo insulin action. Therefore, *ASK1* was directly analyzed as a candidate gene for type 2 diabetes in Pima Indians.

RESEARCH DESIGN AND METHODS

All subjects were part of our ongoing longitudinal study of the etiology of type 2 diabetes among the Gila River Indian Community in Arizona (1). Association with type 2 diabetes was initially assessed in a population-based sample of 3,501 full-heritage Pima Indians. Among these individuals, 1,561 had been diagnosed with type 2 diabetes and 1,940 were nondiabetic at the time of their last exam. Replication for association with type 2 diabetes was assessed in a second population-based sample of 3,723 subjects, most of whom were of mixed-heritage (on average, their reported heritage was one-half Pima and three-quarters American Indian). Among this replication sample, 750 subjects had been diagnosed with type 2 diabetes and 2,973 individuals were nondiabetic at their last exam. Homeostasis model assessment of insulin resistance (HOMA-IR) was analyzed among 2,549 subjects of the full-heritage Pima Indian sample who had available fasting plasma glucose and insulin data measured from the last nondiabetic exam at age ≥15 years. Additionally, more precise measures of diabetes-related quantitative traits were analyzed in 536 nondiabetic Native Americans who had also participated in our 100K GWAS of pre-diabetic traits. *ASK1* mRNA level was assessed using real-time PCR in skeletal muscle biopsies provided by 153 nondiabetic Native Americans involved in the exon array study, among whom 116 subjects had undergone a

TABLE 1
Characteristics of the sample sets used in this study

Sample set	*n*	Full-heritage Pimas	Non–full-heritage Pimas	Diabetes status	Mean age (years)	% Male	Mean BMI (kg/m^2)
Full-heritage Pima Indians	3,501	3,501	0	Diabetic (45%)	48.5 ± 14.1	37	38.5 ± 8.4
				Nondiabetic	31.1 ± 14.5	46	35.7 ± 8.2
Native Americans	3,723	188	3,535	Diabetic (20%)	42.5 ± 14.2	41	38.4 ± 8.8
				Nondiabetic	23.6 ± 10.9	47	33.5 ± 8.4
HOMA-IR analysis*	2,549	2,549	0	Nondiabetic	34.1 ± 13.1	43	35.8 ± 8.3
100K GWAS for pre-diabetic traits†	536	378	158	Nondiabetic	26.8 ± 6.2	59	32.9 ± 6.8
Muscle expression study‡	153	98	55	Nondiabetic	29.4 ± 7.2	70	32.7 ± 6.4
Informative for in vivo insulin action§	116	72	44	Nondiabetic	29.8 ± 7.0	70	32.3 ± 6.1

Data are means ± SD unless otherwise indicated. *Subset of the full-heritage Pima Indian sample. †Subset of both the full-heritage Pima Indian and Native American samples. ‡Subset of the subjects analyzed in the 100K GWAS. §Subset of the subjects with expression data.

hyperinsulinemic-euglycemic clamp to determine their whole-body insulin sensitivity during the same period for which the biopsy was ascertained. Detailed characteristics of these sample sets are given in Table 1. All studies were approved by the tribal council of the Gila River Indian Community and the institutional review board of the National Institute of Diabetes and Digestive and Kidney Diseases.

Metabolic phenotyping. A 75-g oral glucose tolerance test was used to determine diabetes as defined by the criteria of the World Health Organization (5). HOMA-IR was calculated as fasting plasma glucose (mmol/l) × fasting plasma insulin (μU/ml)/22.5. Among subjects studied as inpatients, body composition was estimated by underwater weighing or by total-body dual-energy X-ray absorptiometry (DPX-1; Lunar Radiation) (6). The hyperinsulinemic-euglycemic clamp technique was used to determine insulin-stimulated glucose disposal rate (7). Rates of endogenous glucose production basally and during the clamp were measured using tritiated glucose as previously described (7). The acute insulin response to a 25-g glucose bolus infusion was calculated as previously described (7). Plasma free fatty acid concentrations were measured using a colorimetric assay (Wako Chemicals).

Analysis of *ASK1* expression levels in skeletal muscle. Percutaneous needle biopsies were carried out on the vastus lateralis muscle under local anesthesia with 1% lidocaine after a 12-h overnight fast, and the biopsy specimens were immediately frozen in liquid nitrogen (8). Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and was further purified using a RNeasy Micro Kit (Qiagen, Valencia, CA). cDNA was synthesized using a RETROscript Kit (Ambion, Austin, TX). *ASK1* expression levels were quantified using TaqMan real-time PCR with an ABI PRISM 7700 system (Applied Biosystems, Foster City, CA) using the specific probe and primers for *ASK1* (assay ID: Hs00178726_m1; Applied Biosystems). Each sample was run in triplicate and the *ASK1* expression level was normalized to the mRNA level of cyclophilin A (assay ID: Hs99999904_m1; Applied Biosystems). The relative *ASK1* expression level was determined by the ΔΔCt method according to the manufacturer's protocol (Applied Biosystems).

Sequencing and genotyping. Genotyping and quality-control methods for the 100K GWAS for pre-diabetic traits were the same as previously described for the 100K GWAS for type 2 diabetes (9). To identify novel variants in *ASK1*, all exons, exon-intron boundaries, and 1.2 kb of the putative promoter region were sequenced in 30 nondiabetic Pima Indians who were part of the prior genome-wide exon array study. Sequencing was performed using the Big Dye Terminator (Applied Biosystems) on an automated DNA capillary sequencer (model 3730XL; Applied Biosystems). In addition to six variants identified by sequencing and the SNP providing suggestive evidence for association with insulin action in the prior GWAS, 24 additional database SNPs were directly selected from NCBI dbSNP (Build 36) to provide a denser coverage of the unsequenced intronic regions. The 24 dbSNPs spanned the entire *ASK1* locus with an inter-marker distance of 10 ± 7 kb (mean ± SD) and had an average reported heterozygosity >10%. All these 31 SNPs were genotyped in 1,500 Pima Indians to determine the linkage disequilibrium (LD) pattern across *ASK1* and select tag SNPs. Genotyping was performed by SNPlex (Applied Biosystems) after the manufacturer's protocol.

Statistical analysis. Statistical analyses were performed using SAS software version 9.1 (SAS Institute, Cary, NC). The association of traits with genotype was assessed with an additive model by assigning a numeric variable (0, 1, or 2) to the genotype based on the number of a given allele for each individual. Logistic regression was used to assess the association of genotype with type 2 diabetes, whereas linear regression was used for analysis of continuous variables. Both logistic and linear models were fit with the generalized estimating equation procedure to account for family membership and other potential confounding variables (10). In the replication group, which included many individuals of mixed heritage, the individual estimate of European admixture was also used as a covariate; these estimates were derived by the method of Hanis et al. (11) from 39 markers with large difference in allele frequency between populations (12). A combined test of association with type 2 diabetes for the full-heritage and replication groups was conducted by the inverse variance method (13). The LD pattern was evaluated by Haploview (version 3.32). Tag SNPs were selected using the Tagger algorithm (14) among those with a minor allele frequency >0.05 with a pairwise r^2 ≥0.80 taken as indicative of redundancy.

RESULTS

Sequencing of the exons, exon-intron boundaries, and promoter region of *ASK1* in 30 nondiabetic Pima Indians identified six SNPs, none of which were novel (supplementary Table 1, available in an online appendix at http://diabetes.diabetesjournals.org/cgi/content/full/db09-1700/DC1). A total of 31 variants (six identified by sequencing, 1 from the prior GWAS, and 24 selected from dbSNP; supplementary Table 1) were genotyped in 1,500 Pima Indians to determine the LD structure across *ASK1*. All of these SNPs were in the Hardy-Weinberg equilibrium, and eight tag SNPs were then selected to capture all the common variants (minor allele frequency >0.05) with a pairwise r^2 ≥0.8 (Fig. 1).

The eight tag SNPs were initially genotyped in a population-based sample of 3,501 full-heritage Pima Indians to determine their association with type 2 diabetes (Table 2; quality-control information provided in supplementary Table 2). Three of the tag SNPs were significantly associated with type 2 diabetes (rs35898099, P = 0.003, odds ratio [95% CI] 1.27 [1.08–1.47]; rs1570056, P = 0.007, 1.19 [1.05–1.36]; rs7775356, P = 0.04, 1.14 [1.01–1.28]); adjusted for age, sex, and birth year; Table 2). These three SNPs were further genotyped in a second population-based sample of Native Americans (n = 3,723). rs35898099 was also nominally associated with type 2 diabetes in this second sample (P = 0.04, 1.22 [1.01–1.47], adjusted for age, sex, birth year, and Pima heritage; Table 2). Analyzing the combined samples (n = 7,224) provided significant associations with type 2 diabetes (adjusted P = 0.0004–0.04, odds ratio 1.11–1.24; Table 2) for all three SNPs. To determine if variants in this gene associate with type 2 diabetes in other ethnic groups, SNPs across *ASK1* that were analyzed in the DIAGRAM meta-analysis of Caucasians (15) were examined. rs1570056 also showed nominal association with type 2 diabetes in the DIAGRAM meta-analysis (Z statistic based, P = 0.027) and the risk allele is consistent with the Pimas' risk allele (fixed-effect model,

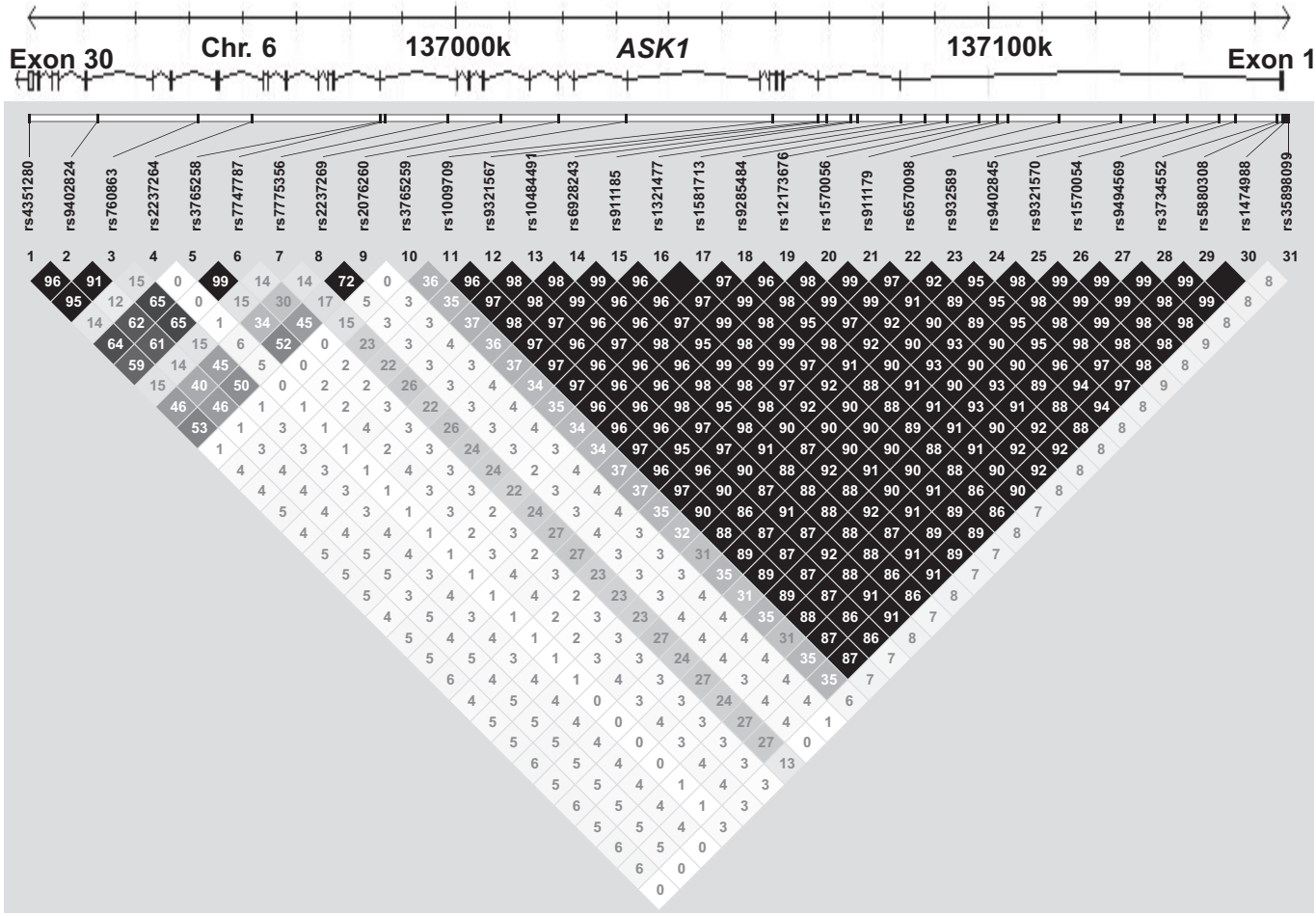

FIG. 1. Relative position and LD pattern of the 31 variants across the *ASK1* locus in Pima Indians. LD is shown as r^2 and the eight tag SNPs (no. 1, 5, 7, 8, 9, 10, 20, and 31) were selected by Tagger algorithm ($r^2 \geq 0.8$, minor allele frequency >0.05).

$P = 0.06$, odds ratio [95% CI] 1.06 [1.00–1.12] per copy of the C allele; Table 2). When the DIAGRAM results for rs1570056 are combined with those from the Pima studies, the summary odds ratio is 1.07 per copy of the C allele (95% CI 1.02–1.13, $P = 0.008$). rs35898099, which was associated with type 2 diabetes in both Native American samples, was not included in the DIAGRAM study or HapMap CEU database. By sequencing 90 Caucasian subjects, we find that rs35898099 is also common in Caucasians (minor allele A frequency = 0.31); however, its association with diabetes in this population remains to be determined. We also performed a conditional analysis for rs1570056 and rs35898099 to assess the possible relevance between their associations with type 2 diabetes in Pima studies. In the full-heritage Pima study, both of the SNPs still showed significant associations with type 2 diabetes conditional on the effect of each other ($P = 0.037$ and 0.02, for rs1570056 and rs35898099, respectively), indicating their effects on type 2 diabetes may be somewhat independent in the full-heritage Pima population. However, when the Native American sample was combined with the full-heritage Pima sample, rs35898099 but not rs1570056 was significantly associated with type 2 diabetes conditional on the effect of the other SNP ($P = 0.001$ for rs35898099 conditional on rs1570056; $P = 0.268$ for rs1570056 conditional on rs35898099).

We further investigated whether the diabetes-associated SNPs were associated with quantitative traits that predict this disease. Among the full-heritage Pima Indians who had available HOMA-IR information when they were nondiabetic ($n = 2{,}549$), rs1570056 was associated with HOMA-IR, where individuals carrying the diabetes-risk allele (C) were more insulin resistant ($P = 0.027$, adjusted for age, sex, and BMI; data not shown). The relationship with insulin resistance was investigated further by analyzing 536 nondiabetic Native Americans who had undergone the hyperinsulinemic-euglycemic clamp, where subjects carrying the diabetes risk allele (C) had decreased rates of insulin-stimulated glucose disposal ($P = 0.02$, adjusted for age, sex, percent body fat, family membership, and Pima heritage; Table 3). The association with insulin resistance was also reflected by the increased plasma insulin levels (adjusted $P = 0.04$ for both fasting and 2-h insulin) during an oral glucose tolerance test. In contrast, no significant difference in percent body fat or measure of acute insulin secretion, by genotype of rs1570056, was observed (Table 3). Similarly, no significant association with rates of endogenous glucose production basally or during the clamp was observed for rs1570056 (Table 3); this suggests that *ASK1* has no specific effect on hepatic insulin resistance. Measures of fasting plasma free fatty acid concentration were available on a small number ($n = 204$) of individuals, and the mean levels were comparable among the different genotype groups for rs1570056 ($P = 0.91$, data not shown).

To determine the potential mechanism whereby SNPs within *ASK1* influence insulin resistance and type 2 diabe-

tes, *ASK1* expression levels were determined by quantitative real-time PCR in muscle cDNA isolated from 153 nondiabetic Native Americans. Adjusted for age, sex, percent body fat, and Pima heritage, the diabetic risk allele (C) of rs1570056 was significantly associated with reduced *ASK1* expression ($P = 0.003$, $r = -0.22$, $n = 153$; Fig. 2). Furthermore, among 116 of these nondiabetic subjects who had undergone the hyperinsulinemic clamp at the time of their biopsy, *ASK1* expression levels were positively correlated with in vivo insulin action ($P = 0.02$, $r = 0.23$, adjusted for age, sex, percent body fat, and Pima heritage; Fig. 3).

DISCUSSION

Although insulin resistance is a predominant clinical feature of type 2 diabetes, most of the type 2 diabetes risk loci identified in prior GWASs appear to affect insulin secretion, but not insulin sensitivity (16). A key finding of the present study is that variations in *ASK1* (tagged by rs1570056 in Pima Indians) increase risk for both in vivo insulin resistance and type 2 diabetes, where the risk alleles are further associated with reduced *ASK1* expression in skeletal muscle and lower *ASK1* expression levels are correlated with reduced insulin sensitivity in vivo. It remains unclear why rs1570056 was associated with type 2 diabetes in the initial population-based sample of full-heritage Pima Indians and the DIAGRAM Caucasians but not the second population-based sample of Native Americans that included mixed-heritage individuals. However, the summary estimate of the odds ratio from both Pima groups and from DIAGRAM was 1.07, and the power of the second population sample is modest for effects of this magnitude, even with 3,700 individuals genotyped. We estimate the power of this second sample to detect an odds ratio of 1.07 given the allele frequency of 0.32 is ~20% at $P < 0.05$ (17). The association for type 2 diabetes did not reach genome-wide significance ($P < 5 \times 10^{-8}$) even in the combined analysis; however, the associations with measures of insulin action and with expression levels suggest that these genetic effects are biologically meaningful, despite the lack of genome-wide significance. The role of rs35898099, which was associated with type 2 diabetes in both the full-heritage Pima Indian and Native American samples, but was not associated with *ASK1* expression, deserves further investigation in other ethnic groups.

Our expression study in skeletal muscle showed the insulin resistance risk allele of rs1570056 was associated with reduced *ASK1* expression and the lower expression level of *ASK1* predicted decreased in vivo insulin action. This finding is consistent with Yang et al. (18), who found that ASK1 (also known as *MAP3K5*) expression in human subcutaneous adipose tissue was positively correlated with in vivo glucose disposal rate. Moreover, they also found the *ASK1* expression was inversely associated with adipose cell mass. Our expression study in skeletal muscle did show a negative correlation of the *ASK1* expression with BMI and percent body fat (adjusted $P = 0.004$ and 0.006, $r = -0.23$ and -0.22, for BMI and percent body fat, respectively; supplementary Fig. 1). Furthermore, we performed real-time PCR for *ASK1* in subcutaneous adipocytes isolated from 77 nondiabetic Pima Indians and confirmed the negative correlation between BMI or percent body fat and *ASK1* expression (adjusted $P = 3.6 \times 10^{-6}$ and 3.3×10^{-7}, $r = -0.50$ and -0.54, for BMI and percent body fat, respectively; supplementary Fig. 2).

TABLE 2
Association of *ASK1* tag SNPs with type 2 diabetes in the Pima studies (full-heritage Pima Indian, Native American, and combined samples), the DIAGRAM study, and the combined analysis of full-heritage Pimas, Native Americans, and DIAGRAM Caucasians (meta-analysis)

	Full-heritage Pima Indian ($n = 3,501$)			Native American ($n = 3,723$)			Combined ($n = 7,224$)			DIAGRAM ($n = 10,128$)			Meta-analysis	
SNP (A1/A2)	A1 (%) (D/ND)	*P*	OR (95% CI)	A1 (%) (D/ND)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P**	*P*†	OR (95% CI)†	*P*	OR (95% CI)
rs4351280 (A/G)	0.85/0.82	0.08	1.15 (0.98–1.35)						0.52	0.34	0.96 (0.88–1.05)		
rs3765258 (G/A)	0.16/0.16	0.67	1.03 (0.88–1.20)										
rs7775356 (T/A)	0.46/0.44	0.04	1.14 (1.01–1.28)	0.46/0.43	0.11	1.13 (0.97–1.31)	0.009	1.13 (1.03–1.24)	0.22	0.21	0.96 (0.90–1.02)	0.68	1.01 (0.96–1.06)
rs2237269 (G/C)	0.83/0.83	0.76	1.02 (0.88–1.19)						0.78	0.73	0.99 (0.90–1.07)		
rs2076260 (T/C)	0.79/0.78	0.43	1.06 (0.92–1.22)						0.77	0.70	0.98 (0.91–1.07)		
rs3765259 (T/C)	0.28/0.26	0.37	1.06 (0.93–1.20)										
rs1570056 (C/T)	0.31/0.27	0.007	1.19 (1.05–1.36)	0.31/0.33	0.97	1.00 (0.85–1.16)	0.04	1.11 (1.01–1.22)	0.026	0.06	1.06 (1.00–1.12)	0.008	1.07 (1.02–1.13)
rs35898099 (G/A)	0.83/0.80	0.003	1.27 (1.08–1.47)	0.80/0.79	0.04	1.22 (1.01–1.47)	0.0004	1.24 (1.10–1.40)					

Odds ratio (OR) is expressed as per copy of allele 1. *P* values were adjusted for age, sex, and birth year. For the Native American sample, *P* values were also adjusted for the individual estimate of European admixture. For DIAGRAM study, both *Z statistic–based *P* value and †fixed-effect model *P* value with OR (95% CI) were shown. A1, allele 1; A2, allele 2; D, diabetic; ND, nondiabetic.

TABLE 3
Associations of rs1570056 (C/T) with diabetes-related traits in the nondiabetic Native Americans who had been metabolically phenotyped

	Phenotype			
	CC	CT	TT	*P*
Female/male (*n*)	24/29	73/98	113/169	
Age (years)	27 ± 0.9	27 ± 0.5	27 ± 0.4	
Percent body fat*†‡§	32.3 ± 1.2	31.8 ± 0.6	32.2 ± 0.5	0.67
BMI (kg/m^2)*†‡§	32.6 ± 0.8	32.6 ± 0.5	33.3 ± 0.4	0.53
Fasting plasma glucose (mg/dl)*†‡§‖	89.1 ± 1.4	88.3 ± 0.7	89.0 ± 0.6	0.59
2-h plasma glucose (mg/dl)*†‡§‖	131.8 ± 4.2	121.1 ± 2.4	121.0 ± 1.8	0.07
Log_{10} fasting plasma insulin (μU/ml)*†‡§‖	1.60 ± 0.03	1.53 ± 0.02	1.54 ± 0.01	0.04
Log_{10} 2-h plasma insulin (μU/ml)*†‡§‖	2.26 ± 0.04	2.17 ± 0.03	2.15 ± 0.02	0.04
Endogenous glucose production basally (mg · kg EMBS^{-1} · min^{-1})*†‡§‖	1.91 ± 0.03	1.89 ± 0.02	1.91 ± 0.01	0.66
Log_{10} insulin-stimulated glucose disposal (mg · kg EMBS^{-1} · min^{-1})*†‡§‖	0.52 ± 0.02	0.57 ± 0.01	0.56 ± 0.01	0.02
Endogenous glucose production during the clamp (mg · kg EMBS^{-1} · min^{-1})*†‡§‖	0.39 ± 0.05	0.30 ± 0.03	0.38 ± 0.02	0.64
Log_{10} acute insulin response (μU/ml)*†‡‖¶	2.30 ± 0.09 (*n* = 19)	2.32 ± 0.03 (*n* = 85)	2.36 ± 0.02 (*n* = 174)	0.48

Data are raw (unadjusted) means ± SE. *P* values are given for an additive model and are adjusted for the covariates as indicated: *age, †sex, ‡family membership, §Pima heritage, ‖percent body fat, and ¶insulin-stimulated glucose disposal rate. The analysis of acute insulin response was restricted to 278 full-heritage Pima Indians who were normal glucose tolerant, and the number of the subjects for each genotypic group is shown in the corresponding parentheses. The diabetic risk allele (C) is shown in italics. EMBS, estimated metabolic body size.

Because the correlation between *ASK1* expression and in vivo insulin action persisted despite control for adiposity, this suggests obesity may not fully account for the insulin resistance caused by lower *ASK1* expression.

Skeletal muscle and adipose tissues are two major insulin-responsive tissues, where insulin increases glucose uptake not only through stimulating GLUT4 translocation to the cell surface but also by increasing the intrinsic activity of GLUT4 (19). As the highest module of MAP kinase cascade, ASK1 may influence both translocation and activation of GLUT4 by activating its downstream MAP2K4/MAP2K7-JNK and MAP2K3/MAP2K6-p38 pathways (20). GLUT4 translocation is regulated by the IRS1-PI3K-Akt signaling pathway (21). JNK activation has been suggested to impair insulin signaling by decreasing insulin-stimulated tyrosine phosphorylation of IRS1 (22). Imoto et al. (23) reported that over-activation of ASK1 in human hepatoma cells impaired insulin signaling and partly mediated tumor necrosis factor (TNF)-α–induced insulin resistance by activating JNK. In this case, reduced ASK1 expression seems likely to promote GLUT4 translocation and improve insulin sensitivity. This hypothesis is not supported by our findings. However, the report by Imoto et al. was based on a hepatoma cell study and thus may not represent what happens in muscle or fat cells in vivo. On the other hand, although GLUT4 translocation is a prerequisite for insulin action in muscle and fat tissue (24,25), evidence suggests that insulin-stimulated glucose transport still needs the activation of GLUT4 (26–28). The activation of p38 MAPK is thought to be involved in the insulin-induced enhancement of intrinsic GLUT4 activity (28–32). For instance, inhibition of p38 by diverse inhibitors suppresses glucose transport but not GLUT4 translocation in both mouse adipocytes and myotubes (28–31). In contrast, activation of p38 by a specific activator stimulates p38 phosphorylation and increases glucose transport in skeletal muscle cells (32). In this aspect, reduced *ASK1* expression may decrease the phosphorylation and inhibit

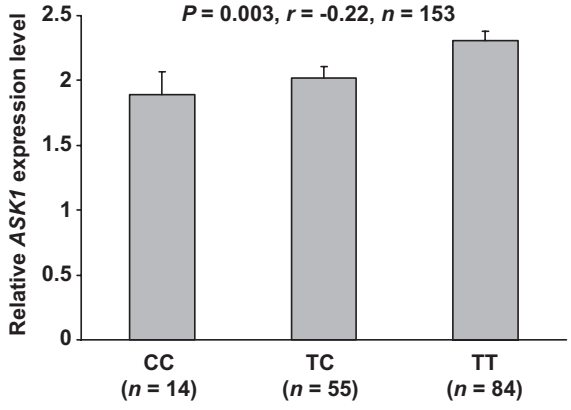

FIG. 2. Association of rs1570056 with *ASK1* expression in skeletal muscle from 153 nondiabetic Native Americans. Data were raw means ± SE. *P* value was calculated under an additive model and adjusted for age, sex, percent body fat, and Pima heritage. Before the analysis, the relative *ASK1* expression levels were logarithmically transformed to approximate the normal distribution.

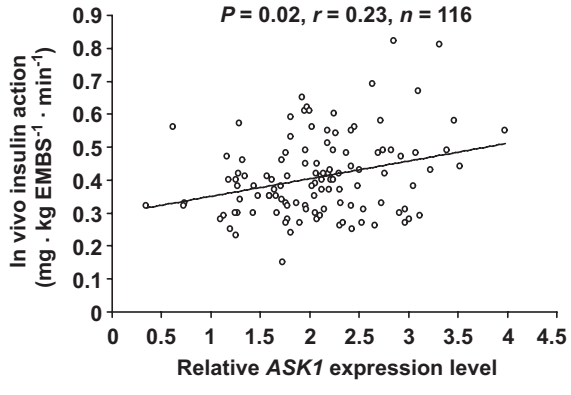

FIG. 3. Positive correlation between *ASK1* expression in skeletal muscle and in vivo insulin action (insulin-stimulated glucose disposal). Before the analysis, both in vivo insulin action (insulin-stimulated glucose disposal) and the relative *ASK1* expression levels were logarithmically transformed to approximate the normal distribution. *P* value was adjusted for age, sex, percent body fat, and Pima heritage. EMBS, estimated metabolic body size.

the activation of p38, which may diminish GLUT4 activity at least on skeletal muscle cell surface. This hypothesis is in agreement with the positive correlation between *ASK1* expression and insulin action observed in our study, suggesting ASK1 may influence insulin action in skeletal muscle mainly through the effect on the p38 MAP kinase pathway rather than on the JNK pathway.

The region of LD tagged by rs1570056 is large and spans the *ASK1* promoter through intron 5, making it is difficult to determine the causative variant(s) underlying these associations. Based on sequence inspection, one potential causative variant was rs5880308, a short tandem repeat variant in the core promoter region of *ASK1* that was predicted to affect a TFIIB binding site (MapInspector at www.genomatix.de). However, we conducted a luciferase assay that did not show any significant difference in reporter gene activity between the two constructs carrying different alleles (data not shown), suggesting that this variant is not functional. These results are consistent with the study of Arning et al. (33). Thus, additional functional studies are required to determine the true causative variant that affects the *ASK1* expression.

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