A genetic variant at the fatty acid-binding protein aP2 locus reduces the risk for hypertriglyceridemia, type 2 diabetes, and cardiovascular disease

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Obesity and the associated pathologies including dyslipidemia, insulin resistance, type 2 diabetes, and cardiovascular disease constitute a major threat to global human health. Yet, the genetic factors that differentially predispose individuals to this cluster of pathologies are unclear. The fatty acid-binding protein aP2 is a cytoplasmic lipid chaperon expressed in adipocytes and macrophages. Mice with aP2 deficiency are partially resistant to obesity-induced insulin resistance and type 2 diabetes, have lower circulating triglycerides, and exhibit marked protection against atherosclerosis. Here, we demonstrate a functionally significant genetic variation at the aP2 locus in humans that results in decreased adipose tissue aP2 expression due to alteration of the CAAT box/enhancer-binding protein binding and reduced transcriptional activity of the aP2 promoter. In population genetic studies with 7,899 participants, individuals that carry this T-87C polymorphism had lower serum triglyceride levels and significantly reduced risk for coronary heart disease and type 2 diabetes compared with subjects homozygous for the WT allele. Taken together, our results indicate that reduction in aP2 activity in humans generates a metabolically favorable phenotype that is similar to aP2 deficiency in experimental models.

The predominant FABP present in adipocytes is aP2, with mal1 being the minor isoform (7). In contrast, both isoforms are expressed at similar levels in activated macrophages (6) and regulated by a variety of inflammatory and metabolic mediators (8, 9). The precise physiological role of these small proteins has been recognized only upon the development of genetic models to examine their function in mice (4, 10, 11). In these experimental models, deficiency of aP2 results in lowered triglycerides, increased insulin sensitivity when made obese, and protection against atherosclerosis in models of hypercholesterolemia (6, 12, 13). The impact of aP2 deficiency on atherosclerosis is predominantly the result of its action in macrophages (6). The contribution of these cell types to FABP effects on systemic glucose and lipid metabolism has not yet been fully dissected.

Taken together, FABPs, particularly aP2, serve as a locus of integration for metabolic and inflammatory pathways and play a critical role in systemic metabolic homeostasis and cardiovascular disease in experimental mice models (4, 11). Because aP2 expression and regulation exhibits striking similarities between rodents and humans (8, 14), it is feasible to postulate that genetic variations at this locus might influence similar metabolic outcomes in humans provided that they regulate the expression of aP2. To test this hypothesis, we examined sequence alterations at the aP2 locus, evaluated functional consequences and tested relevance to risk of hypertriglyceridemia, type 2 diabetes, and cardiovascular disease, the three traits that are closely linked to aP2 action, in human populations.

Results

SNP Discovery. The aP2 gene, which has been mapped to chromosome 8q21 (Mendelian Inheritance in Man number 600434), consists of four exons, and encodes a 132-aa protein (15, 16). The locus spans ~7 kb of genomic DNA. To determine the potential genetic variation(s) at the aP2 locus, we determined the genomic DNA sequence for the promoter and coding regions in randomly selected 96 men and women of primarily Caucasian descent. Through these studies, we identified and confirmed five distinct SNPs. The summary of these SNPs is shown in Table 1. Of the five, two of the sequence variants, C2600T and G4356C, were previously reported in National Center for Biotechnology Information SNP database as rs8192688 and rs1051252, respectively. All of the SNPs were outside the coding regions except for G4356C, which is a silent variant on exon 4 of aP2.
Table 1. The sequence variations found in aP2 gene

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Position, bp</th>
<th>rs no.</th>
<th>Allele freq., %</th>
</tr>
</thead>
<tbody>
<tr>
<td>T → C</td>
<td>-87</td>
<td></td>
<td>4.69</td>
</tr>
<tr>
<td>C → T</td>
<td>2600</td>
<td>8192688</td>
<td>16.67</td>
</tr>
<tr>
<td>T → C</td>
<td>2613</td>
<td></td>
<td>0.52</td>
</tr>
<tr>
<td>Ins G</td>
<td>4265</td>
<td></td>
<td>16.67</td>
</tr>
<tr>
<td>G → C</td>
<td>4356</td>
<td>1051252</td>
<td>0.52</td>
</tr>
</tbody>
</table>

The promoter and coding region of the aP2 gene, including the intron/exon boundaries, were sequenced in 96 male and female subjects whom were predominantly Caucasian. The list of sequence variations observed in this group is shown. When available, the existing SNP database presence is indicated with reference number (rs no.). The nucleotide position shown is based on reverse-strand human aP2 sequence with respect to transcriptional initiation site.

We also identified a unique variant at position -87, which was a base alteration of T to C (T-87C) in the 5′ promoter region of human aP2 gene and was in moderate linkage disequilibrium with C2600T and InsG4265 SNPs (D’ = 0.6995 and 0.6912, respectively). T-87C is predicted to be located at the CAAT box/enhancer-binding protein (C/EBP) binding site (Fig. 1a). Because this site is critically important for the expression and regulation of murine aP2 gene (17), we examined the potential functional consequences of this genetic variation and its association with metabolic disease risk in humans.

C/EBPα Binding to WT and T-87C Variant of the aP2 Promoter. Because this SNP alters the cognate C/EBP recognition site, we first examined potential alterations in the ability of C/EBPα to bind to this site on the human aP2 promoter. A DNA fragment containing the target promoter sequences of aP2 (nucleotides −194 to −16) was radiolabeled to examine binding of C/EBPα protein to its cognate DNA. In control experiments, a dose-dependent binding of C/EBPα was observed with an increasing amount of protein (Fig. 1b). The specific interaction of C/EBPα was determined by the formation of a supershift with the addition of a C/EBPα antibody to the reaction. The binding of C/EBPα also was effectively competed by the addition of unlabeled oligonucleotide competitors that contain the C/EBPα binding (Fig. 1b). Having established these controls, we then tested whether the T-87C variant differed in its ability to interact with C/EBPα protein. We performed EMSA with C/EBPα and its cognate DNA by including increasing amounts of unlabeled competing DNA oligonucleotides with the WT or variant aP2 promoter sequences (Fig. 1c). These experiments demonstrated that the DNA sequence harboring the T-87C variant exhibited significantly reduced competitive-binding capacity to C/EBPα compared with WT aP2 promoter sequence (Fig. 1c). At all doses of competition, there was ≥50% reduction in the ability of the mutant sequence to bind to C/EBPα compared with that of the WT.

Activity of the WT and T-87C Variant of the aP2 Promoter. Next, we examined the impact of the T-87C variant on the activity of the aP2 promoter. HEK-293 cells were cotransfected with reporter constructs where luciferase gene expression was driven by either WT or mutant aP2 promoter alleles and control or C/EBPα expressing vector. As shown in Fig. 2, the activity of the WT promoter could be detected, albeit at relatively low levels in these
cells in the absence of exogenously added C/EBPα. This low baseline promoter activity level was similar between the two genotypes. When these experiments were performed in the presence of different levels of exogenously expressed C/EBPα, a 3- to 10-fold increase was observed in the WT promoter activity. However, there was only a minimal (1.5-fold) increase in the mutant aP2 promoter upon addition of C/EBPα. These experiments clearly demonstrate that the T-87C variant reduced the binding ability of C/EBPα to its recognition site at this location and significantly impaired transcriptional activation of aP2 promoter by C/EBPα in cultured cells.

Genotype–Phenotype Studies in Population Cohorts. Based on the results of our functional characterization, we expanded our studies on this SNP to two large ongoing prospective studies designed to identify genetic and lifestyle predictors of chronic disease. The genotype at nucleotide −87 of the aP2 promoter was determined in a total of 7,899 subjects (3,050 female and 4,849 male) from the Nurses’ Health Study and Health Professionals Follow-up Study cohorts described below. The heterozygosity prevalence for the T-87C variant was 4.3% with an allele frequency of 2.2%. Compared with WT, par-

cipants heterozygous for the aP2 polymorphism had an odds ratio for CHD of 0.66 (95% confidence interval: 0.46, 0.97; P = 0.03) (Fig. 3). Hence, the presence of this aP2 variant was associated with significantly reduced risk for coronary heart disease.

Because FABPs regulate systemic glucose metabolism and insulin action in obese mice, we further explored the association between the aP2 variant and risk of diabetes. We found a marginally significant reduced risk of diabetes for carriers of aP2 variant (odd ratio = 0.75; 95% confidence interval: 0.55, 1.03; P = 0.07). In a stressed metabolic state, the effect of the variant may be greater. In fact, aP2 deficiency in mice exhibits its impact on insulin action and type 2 diabetes only in the presence of diet-induced or genetic obesity (18). Therefore, we asked whether this paradigm might be similar in humans and examined diabetes risk only in the obese participants. Among the 1,156 obese [body mass index (BMI) ≥30 kg/m²] female and male subjects the odds of diabetes (odds ratio = 0.52; 95% confidence interval: 0.29–0.93; P = 0.03) was significantly reduced among carriers of the aP2 promoter polymorphism (Fig. 4).

Impact of −87 Variant on aP2 Gene Expression in Adipose Tissue of Carriers. Our experiments by using reporter assays and EMSA indicated that the SNP at −87 of the aP2 promoter has a negative impact on the promoter activity and expression of the aP2 gene (Figs. 1 and 2). Moreover, the three predominant phenotypic features of the aP2-deficient mice, reduced triglycerides, reduced

Table 2. Genotype frequencies

<table>
<thead>
<tr>
<th>Gender</th>
<th>WT/WT</th>
<th>WT/T-87C</th>
<th>T-87C/T-87C</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>2,910 (95.41)</td>
<td>139 (4.56)</td>
<td>1 (0.03)</td>
<td>3,050</td>
</tr>
<tr>
<td>Male</td>
<td>4,645 (95.79)</td>
<td>202 (4.17)</td>
<td>2 (0.04)</td>
<td>4,849</td>
</tr>
<tr>
<td>Total</td>
<td>7,555 (95.65)</td>
<td>341 (4.32)</td>
<td>3 (0.04)</td>
<td>7,899</td>
</tr>
</tbody>
</table>

Frequency of the T-87C genotypes observed in the total population studied. Upon identification by sequencing, the T-87C SNP was studied utilizing a TaqMan-based screen. Allele distribution is shown in absolute numbers and percentages (in parentheses).

Table 3. Least-square means for lipid, inflammatory, and glycemic parameters by aP2 genotype

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT/WT</th>
<th>WT/T-87C</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglycerides, mg/dl</td>
<td>176.7</td>
<td>153.6</td>
<td>0.009</td>
</tr>
<tr>
<td>CRP, mg/liter</td>
<td>2.81</td>
<td>2.89</td>
<td>0.83</td>
</tr>
<tr>
<td>IL-6, pg/ml</td>
<td>2.84</td>
<td>4.97</td>
<td>0.50</td>
</tr>
<tr>
<td>TNF, pg/ml</td>
<td>1236</td>
<td>1187</td>
<td>0.25</td>
</tr>
<tr>
<td>HDL-C, mg/dl</td>
<td>137.0</td>
<td>133.9</td>
<td>0.27</td>
</tr>
<tr>
<td>LDL-C, mg/dl</td>
<td>50.9</td>
<td>51.1</td>
<td>0.90</td>
</tr>
<tr>
<td>ApoB, mg/dl</td>
<td>114.4</td>
<td>109.6</td>
<td>0.06</td>
</tr>
<tr>
<td>Hemoglobin A1C, %</td>
<td>6.29</td>
<td>6.17</td>
<td>0.32</td>
</tr>
</tbody>
</table>

The multivariate model is controlled for age, BMI, activity, smoking, calories, saturated fat, transfat, fasting hours, and study groups. *P = 0.03. For triglycerides, we only used the participants with fasting samples. For each blood parameter the number of samples with available data ranged from 1,167 (for IL-6 and TNFR1) to 3,716 for CRP.
type 2 diabetes, and coronary heart disease, also were captured among the human carriers of the aP2–87 SNP. Therefore, a critical and definitive link between these observations lies with the demonstration of altered in vivo aP2 expression because of aP2–87 in humans.

To address this critical question, we genotyped an additional 1,247 subjects from which adipose tissue needle-biopsy samples has been collected along with blood samples (19) for a nonfatal MI case-control study in Costa Rica. Among these subjects, we identified 31 individuals heterozygous for aP2–87 and matched WT carriers according to age, sex, dietary habits, and region of residence. After RNA isolation and cDNA synthesis from these samples, we established a real-time quantitative PCR assay for human aP2 gene. The assay was further validated with a mixing experiment by pooling RNA from aP2 expressing (adipose) and nonexpressing tissues at precalculated ratios followed by RT-PCR analyses. In this experiment, the quantitative experimental measurement was in perfect agreement with the aP2 ratio in premixed samples (r = 0.98). After this validation, we examined the levels of aP2 expression in tissue samples obtained from subjects with the T-87C compared with WT controls. As shown in Fig. 5, subjects carrying the T-87C polymorphism had a 63% reduction in adipose tissue aP2 mRNA expression levels compared with those with the WT allele (0.078 versus 0.21 respectively; P = 0.017). Hence, the T-87C is a loss-of-function allele in vivo and results in reduction of adipose tissue aP2 mRNA expression in humans.

**Discussion**

The global prevalence of obesity and the associated metabolic disease cluster is increasing rapidly. In the United States, 65.7% of adults and 16% of children are overweight and 23.7% of adults have metabolic syndrome, an aggregation of comorbidities, including obesity, insulin resistance, dyslipidemias, and hypertension. The presence of metabolic syndrome introduces great risk for coronary events, stroke, type 2 diabetes, and other chronic health problems. These pathological states have strong inflammatory underpinnings (3). Integrated metabolic abnormalities emerge from the action of common pathways to metabolic and inflammatory cells, the prototype example is between adipocytes and macrophages in obesity. Hence, molecular pathways controlling the responses of these cells are of critical importance in understanding the underlying mechanism(s) of metabolic syndrome.

In recent years, results obtained from experimental models demonstrated that FABPs integrate metabolic and immune responses and link the inflammatory and lipid-mediated pathways that are critical in metabolic syndrome (9). In particular, the adipocyte/macrophage FABP isoform, aP2, when deleted in mice demonstrates three major phenotypes: moderately reduced triglyceride levels, protection against type 2 diabetes when the animals were made obese, and reduced atherosclerosis despite hypercholesterolemia (6, 18). Similar to mice, aP2 is expressed in human adipocytes and regulated by differentiation. It is also detected in activated macrophages, and aP2 level is elevated in human atherosclerotic lesions. Interestingly, aP2 expression in macrophages is negatively regulated by statins (20). As directly shown in mice models, reduction in aP2 abundance is metabolically beneficial; if similar in humans then alleles that reduce aP2 expression or activity might confer resistance to some or all of these parameters in humans. In this study, we have searched for polymorphisms associated with regulation of aP2 expression in humans and tested this hypothesis in a large prospective population study. Our studies revealed a functionally significant SNP, T-87C, in the 5′ promoter region of aP2, which disturbs C/EBPα binding and subsequent transcriptional activation of this promoter by C/EBPα. Hence, the T-87C allele represents a “loss-of-function” allele of aP2 in humans. Consistent with this inference, subjects that carry this aP2 allele exhibit reduced serum triglycerides and significantly reduced risk for type 2 diabetes and cardiovascular diseases. In other words, the phenotypic outcome of the T-87C allele in humans is highly reminiscent of the phenotype of aP2 deficiency in the mice. In general, we were able to replicate our findings from our cohort of women and in our cohort of men. However, these subjects were healthy, free-living, mostly Caucasian populations. Confirmation from other populations, especially those with a higher variant allele prevalence is warranted.

A critical piece of evidence often challenging to gather in studies of this nature is to link the presence of a functionally significant genetic variant to a change of function in vivo in the individuals that carry the corresponding allele. In our study, we provide compelling evidence that the presence of T-87C allele results in significantly reduced aP2 gene expression in the adipose tissue of carrier subjects. In this study, we did not have the opportunity to evaluate the potential impact of this variant on macrophage aP2 expression and function. It is possible that this loss-of-function allele might have important effects on these cells that are not captured in this setting. The magnitude of changes in adipose tissue aP2 expression is similar to haploinsufficiency in experimental mice with one targeted aP2 allele (12). Studies on the functional consequences of aP2 haploinsufficiency in mice are rather limited. Interestingly, mice with one
dysfunctional allele of aP2 gene exhibit an intermediary phenotype, at least in lipid and glucose metabolism, upon exposure to a high-fat diet or in genetic obesity (12). The impact of haploinsufficiency has not yet been examined in the context of atherosclerosis and macrophage function (6, 21).

Taken together our studies illustrate that an allele with reduced aP2 activity in free-living mice and, therefore, chemical modification of aP2 activity in humans might represent a desirable preventive or therapeutic strategy against the devastating cluster of diseases such as type 2 diabetes and atherosclerosis.

Materials and Methods
Population. The Nurses’ Health Study cohort is a prospective study of 121,700 women, 32,826 of which (34–69 years of age) were followed biennially to document incident chronic disease. We had available to us for genotyping several previously transfected with the 179-bp WT or mutant aP2 proximal fragment (nucleotides −194 to −16) driving the expression of luciferase gene in the pGL3 luciferase reporter vector (Promega) with or without full-length C/EβPα expression vector. Transfections were performed with varying DNA concentrations at 60–70% cellular confluency by using FuGene6 reagent (Roche Applied Science, Indianapolis). Thirty-six hours after the transfections cells were washed with PBS solution, lysed, and dual luciferase assay (Promega) was performed to determine activity. Renilla luciferase reporter was used as an internal control for transfection efficiency and to normalize the results.

Sequencing, SNP Discovery, and Genotyping. The sequencing of the entire coding and promoter regions of aP2 was carried out with AmpliTaq DNA Polymerase FS dye terminator method by using ABI PRISM 377 DNA Sequencer (Applied Biosystems). The primers used for sequencing were as follows: exon1F: 5′-TgCatCTCCCTCTCCTCCTGTA-3′, exon1R: 5′-CTCTCTATGTTGCACTTCCTC-3′, exon2F: 5′-ACACACACTTTCTCTGACTTgg-3′, exon2R: 5′-gAgTCTTAgCTTgATgTTgC-3′, exon3F: 5′-CCTCTCCTTGACATTgTCTT-3′, exon3R: 5′-AggCATAgCgAgTgAgAgAg-3′, exon4F: 5′-TATCTCCCAACATgAGAC-3′, exon4R: 5′-TATCCCAACATgAGAC-3′. (Integrated DNA Technologies, Coralville, IA). For each sample, sequencing was performed both with forward and reverse primers. For the T-87C promoter polymorphism, all samples were genotyped by using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) in a 384-well format. The 5′ nuclease assay (TaqMan) was used to distinguish the two alleles of the aP2 gene, involving a T→C transition at position −87 from the transcription start site (reverse complement strand). The PCR amplification was carried out on 5–20 ng of DNA by using 1× TaqMan universal PCR master mix (No Amp-erase UNG), 900 nM forward (5′-TCTTTTTAAgATgCTCCAgACATgTGA-3′) and reverse (5′-gCCtgTAATgAgAAATgATTgAAA-3′) primers, 200 nM of the FAM-labeled probe (5′-CATTCCCAACATgTGgTCAT-3′) and 200 nM of the VIC-labeled probe (5′-CTCTCCCAATgTGgTCAT-3′) in a 5-μl reaction, and the polymorphic base is shown underlined. Amplification conditions on a AB 9700 dual plate thermal cycle (Applied Biosystems) were as follows: 1 cycle of 95°C for 10 min, followed by 50 cycles of 92°C for 15 s and 58°C for 1 min. TaqMan primers and probes were designed, on the reverse strand, by using the PRIMER EXPRESS 2.0 oligo design software (Applied Biosystems).

Plasmid Preparation and Gel Shift Assays. A DNA segment of aP2 spanning the region between nucleotides −194 and −16 in the 5′ region of human aP2 promoter was amplified by using primers PromFwd: 5′-CATgGAgCTCTTTATTgCCAgAgAgACC-3′ and PromRev: 5′-gAgTAGCTAggCTgACCCCTCTgAgTTgCC-3′, thereby creating SacI (5′ end) and NheI (3′ end) restriction sites at the ends. After restriction enzyme digestion of the PCR product along with the pGL3 basic vector, ligation was performed by using T4 DNA Ligase (NEB, Beverly, MA). Site-directed mutagenesis was performed with aP2mutFwd: 5′-TgATgTgATgCTCCATgATCCTCC-3′ and aPmutRev: 5′-TgAAgATCCACTATTgCCTTC-3′ by using GeneTailor kit (Invitrogen) and confirmed by sequencing.

The 179-bp human aP2 proximal promoter segment carrying either a WT or mutant allele was used as a DNA probe in EMSA. The EMSA reactions were performed in 30-μl volume containing 400 ng of DNA labeled with 10 μCi [γ-32P]ATP (1 Ci = 37 GBq) and T4 PNK (NEB, Beverly, MA). The protein component, 15 ng of GST-C/EβPα (12 kDa carbonyl-terminal rat C/EβPα fusion protein; ref. 27), was incubated with 0.5 μg poly(d-I-C) and 32P-labeled DNA probe (2 × 106 cpm) in the presence or absence of varying quantities of specific (with or without the T→C alteration) or nonspecific unlabeled oligonucleotide competitors at room temperature for 20 min. At the end of the incubation period, the samples were run on a 4% nondenaturing polyacrylamide gel for 2 h, dried, and exposed to Imaging Screen K (Bio-Rad) for visualization by Molecular Imager FX (Bio-Rad). For super shifting, anti-C/EβPα antibody was used (Santa Cruz Biotechnology).

Promoter Activity Reporter Assays. Promoter activity assays were performed in HEK-293 cells, maintained in DMEM (GIBCO), and supplemented with 10% FBS (HyClone). Cells were cotransfected with the 179-bp WT or mutant aP2 proximal promoter fragment (nucleotides −194 to −16) driving the expression of luciferase gene in the pGL3 luciferase reporter vector (Promega) with or without full-length C/EβPα expression vector. Transfections were performed with varying DNA concentrations at 60–70% cellular confluency by using FuGene6 reagent (Roche Applied Science, Indianapolis). Thirty-six hours after the transfections cells were washed with PBS solution, lysed, and dual luciferase assay (Promega) was performed to determine activity. Renilla luciferase reporter was used as an internal control for transfection efficiency and to normalize the results.

Real-Time RT-PCR. Total RNA was isolated from 50 mg of adipose tissue sample by using TRIzol reagent (Invitrogen). Reverse transcription reaction was carried out with ThermoScript RT-PCR system (Invitrogen). Real-time PCR analysis was performed in a 25-μl final reaction volume with an iCycler iQ Detection System by using iQ SYBR Green Supermix (Bio-Rad). The PCR thermal cycling program was as follows: 2 min 30 s at 95°C for enzyme activation (allowing hot start), 40 cycles of 15 s at 95°C, 30 s at 58°C, and 1 min at 72°C for extension. Melting curve analysis was performed to confirm the real-time PCR products. All quantifications were normalized to the 18S rRNA level in reaction. Primer sequences used were as follows: aP2Fwd: 5′-gCACCACTACCTAgATgggg-3′; aP2Rev: 5′-TggAAgTgAATgCCCTTCCAACACTTgTGgTCAT-3′; 18S Fwd: 5′-gTAACCTCCACATgTTgTGgTCAT-3′, and 18S Rev: 5′-CACATCCACTACCTgTgACgC-3′.

Statistical Analysis. Multivariate linear regression analyses with robust variance were performed to evaluate the association between triglyceride levels and the aP2 polymorphism. We adjusted for age, sex, BMI, activity, smoking, fasting status, and dietary intake of trans and saturated fatty acids and total calorie intake. We used unconditional logistic regression to determine
the odds ratio (95% confidence interval) for CHD and diabetes mellitus for participants with and without the variant allele. The multivariate analyses were controlled for age, sex, smoking, physical activity, and BMI. Further control for other lifestyle characteristics did not appreciably alter the odds ratio estimates.

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