Links Between Insulin Resistance, Adenosine A2B Receptors, and Inflammatory Markers in Mice and Humans

Robert A. Figler,1 Guoquan Wang,2 Susseela Srinivasan,1 Dae Young Jung,3 Zhiyou Zhang,3 James S. Pankow,4 Katya Ravid,5 Bertil Fredholm,6 Catherine C. Hedrick,1 Stephen S. Rich,7 Robert A. Figler,1 Guoquan Wang,2 Susseela Srinivasan,1 Dae Young Jung,3 Zhiyou Zhang,3 James S. Pankow,4 Katya Ravid,5 Bertil Fredholm,6 Catherine C. Hedrick,1 Stephen S. Rich,7 Jason K. Kim,3 Kathryn F. LaNoue,5 and Joel Linden1

OBJECTIVE—To determine the mechanisms by which blockade of adenosine A2B receptors (A2BRs) reduces insulin resistance.

RESEARCH DESIGN AND METHODS—We investigated the effects of deleting or blocking the A2BR on insulin sensitivity using glucose tolerance tests (GTTs) and hyperinsulinemic-euglycemic clamps in mouse models of type 2 diabetes. The effects of diabetes on A2BR transcription and signaling were measured in human and mouse macrophages and mouse endothelial cells. In addition, tag single nucleotide polymorphisms (SNPs) in ~42 kb encompassing the A2BR gene, ADORA2B, were evaluated for associations with markers of diabetes and inflammation.

RESULTS—Treatment of mice with the nonselective adenosine receptor agonist 5'-N-ethylcarboxamidoadenosine (NECA) increased fasting blood glucose and slowed glucose disposal during GTTs. These responses were inhibited by A2BR deletion or blockade and minimally affected by deletion of A1Rs or A2ARs. During hyperinsulinemic-euglycemic clamp of diabetic KKA mice, A2BR antagonism increased glucose infusion rate, reduced hepatic glucose production, and increased glucose uptake into skeletal muscle and brown adipose tissue. Diabetes caused a four- to sixfold increase in A2BR mRNA in endothelial cells and macrophages and resulted in enhanced interleukin (IL)-6 production in response to NECA due to activation of protein kinases A and C. Five consecutive tag SNPs in ADORA2B were highly correlated with IL-6 and C-reactive protein (CRP). Diabetes had a highly significant independent effect on variation in inflammatory markers. The strength of associations between several ADORA2B SNPs and inflammatory markers was increased when accounting for diabetes status.

CONCLUSIONS—Diabetes affects the production of adenosine and the expression of A2BRs that stimulate IL-6 and CRP production, insulin resistance, and the association between ADORA2B SNPs and inflammatory markers. We hypothesize that increased A2BR signaling in diabetes increases insulin resistance in part by elevating proinflammatory mediators. Selective A2BR blockers may be useful to treat insulin resistance. Diabetes 60:669–679, 2011

O
besity and insulin resistance are associated with low-grade systemic inflammation. Proinflammatory mediators produced in adipose tissue (adipokines) that increase insulin resistance include interleukin (IL)-6 (1), C-reactive protein (CRP) (2), and plasminogen activator inhibitor 1 (PAI-1) (3). In addition, insulin resistance due to a high-fat diet causes macrophage accumulation in adipose tissue and M2-like remodeling (4). Endothelial dysfunction is also a hallmark of diabetes because inflammatory mediators activate receptors and transcription factors such as nuclear factor-κB, toll-like receptors, c-Jun NH2-terminal kinase (JNK), and the receptor for advanced glycation end products, which cause systemic endothelial dysfunction (5).

Several studies have linked adenosine receptor blockade with reversal of insulin resistance. Challis et al. reported that adenosine receptor antagonists (6) or degradation of adenosine with adenosine deaminase (7) reverse insulin resistance in skeletal muscle isolated from diabetic animals. After a lengthy delay before the development of bioavailable adenosine receptor antagonists, the A1/A2A orally active antagonist, BW-1433, was found to consistently reverse insulin resistance in obese insulin-resistant Zucker rats (8–10). In these early studies, the effects of adenosine receptor antagonists were attributed to blockade of A1Rs. This idea was corrected when moderately selective blockers of the A2BR were found to lower glucose levels in diabetic mice, an effect that could not be replicated with the selective A1R antagonist 8-cyclopentyl-1,3-dipropylxanthine (11). In mice rendered insulin resistant due to a high-fat diet, ADORA2B gene deletion results in reduced adiposity, reduced liver glycogen, increased energy expenditure, and increased lean body mass (12).

In the current study we confirm that A2BR activation stimulates IL-6 production in macrophages and endothelial cells (ECs) and show that these effects are enhanced in cells derived from diabetic animals. Blockade of A2ARs in diabetic mice reduces hepatic glucose production (HGP) and enhances glucose disposal into skeletal muscle and brown adipose tissue. In addition, diabetes influences the association of single nucleotide polymorphisms (SNPs) in ADORA2B with IL-6 and CRP. These findings suggest that diabetes and one or more SNPs in ADORA2B influence proinflammatory A2BR signaling.
RESEARCH DESIGN AND METHODS

RT-PCR. Total RNA was isolated from ECs or macrophages using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Sense/antisense mouse PCR primers were KC 5′-ctggtaggctgcgccagc-3′/5′-tgggagcactttgatac-3′; IL-6 5′-ctgctgctgggtaaac-3′/5′-tccagcagtcctcagaa-3′; A2AR 5′-ctggtaggctgcgccagc-3′/5′-ctgggagcactttgatac-3′; and A2BR 5′-ctggagctcctctcc-3′/5′-gtgggccagctttac-3′. Sense/antisense human PCR primers were A2AR 5′-agtgcgcaatcctc-3′/5′-actgctctctgagt-3′; A2BR 5′-ggtcttgctctctc-5′-tactggcttgcttcac-3′.

Isolation and culture of human macrophages. Heparinized blood was collected from healthy and diabetic volunteers in accordance with guidance from the University of Virginia Institutional Review Board. Monocytes were isolated using Rosette Sep human monocytes enrichment cocktail ( StemCell Technologies, Takwa, WA) and plated in a tissue culture dish in Dulbecco’s modified Eagle’s medium (DMEM) with 10% autologous serum and 10 ng/ml human macrophage colony-stimulating factor for 3 days. Total RNA was isolated from the differentiated macrophages using TRIzol reagent (Invitrogen). cDNA was synthesized with Iscript cDNA synthesis kit (Bio-Rad) using 1 μg of total RNA. Expression of A2AR, A2BR, and β-actin mRNA levels were measured by quantitative RT-PCR.

Transgenic mice. The University of Virginia Animal Care and Use Committee approved animal studies. Mice with adenosine receptor deletions used in this study were congeneric to C57BL/6 and were created as described previously: A2AR+/− (13), A2AR−/− (14), and A2BR−/− (15). Some studies used diabetic B6. Cg-m1Lept/J (db/db) with diabetic C57BL/6J controls or diabetic KK.A1R+/− (KK-A1R) with less diabetic KK.H1J (KK-a1R) controls as identified in the figures. Feeding a high-fat diet (50% calories from fat, Harlan TD90075; 125 mg/kg ATL-801) for 10 weeks was used to produce insulin resistance in C57BL/6 mice.

In vivo assessment of insulin sensitivity. A 2-h hyperinsulinenic-euglycemic clamp was performed in conscious mice to assess insulin action and glucose metabolism in individual organs. At 4 to 5 days before clamp experiments, mice were anesthetized, and an indwelling catheter was inserted in the right jugular vein. On the day of clamp experiments, a three-way connector was attached to the catheter to intravenously deliver solutions (e.g., glucose, insulin). After overnight fast (~15 h), a 2-h hyperinsulinenic-euglycemic clamp was conducted in conscious mice with a primed (150 mU/kg body wt) and continuous infusion of human regular insulin (Humulin; Eli Lilly, Indianapolis, IN) at a rate of 2.5 mU/kg/min to raise plasma insulin within a physiological range. Blood samples (20 μl) were collected at 20-min intervals for the immediate measurement of plasma glucose concentration, and 20% glucose was infused at variable rates to maintain glucose at basal concentrations. Basal and insulin-stimulated whole body glucose turnover were estimated with a continuous infusion of [3H]glucose (PerkinElmer, Boston, MA) for 2 h before the clamps (0.05 μCi/min) and throughout the clamps (0.1 μCi/min), respectively. All infusions were performed using the microdialysis pumps (CMA/Microdialysis, North Chelmsford, MA). To estimate insulin-stimulated glucose uptake in individual tissues, 2-deoxy-D-[1-14C]glucose was administered as a bolus (10 μCi) at 75 min after the start of clamps. Blood samples were taken before, during, and at the end of clamps for the measurement of plasma [3H]glucose, [3H]2,3-deoxy-D-[1-14C]glucose concentrations, and/or insulin concentrations. At the end of the clamps, mice were killed and tissues were taken for biochemical and molecular analysis. KKA’ mice were treated with 20 mg/kg ATL-801 administered by oral gavage four times at 12-h intervals with the last dose given 90 min before the clamp.

Association of human ADORA2B SNPs with phenotypic markers. The Multi-Ethnic Study of Atherosclerosis (MESA) is a prospective cohort study designed to study the progression of subclinical cardiovascular disease, consisting of 6,814 men and women aged 45–85 years who were free of clinical cardiovascular disease at entry. The participants were recruited from six U.S. communities. The sampling procedures have been described previously (16), and the protocol and research methods are available on the MESA Web site (http://www.mesa-nhlbi.org). A subcohort of 2,580 MESA subjects (913 men and 1,667 women from each of the four ethnic groups) was randomly selected from subjects who gave informed consent for genetic studies. All phenotypic data reported in this study were collected at the first MESA examination according to Declaration of Helsinki principles. Details of phenotyping and genotyping procedures are described in Supplementary Data.

Evaluation of SNP-diabetes interactions. For all SNPs, genotype-specific means and variances for each quantitative phenotype were estimated overall and within strata (ethnic group and diabetes status). Empirical P values were determined by permutation. A label-swapping approach was used, in which each SNP genotype is permuted for each phenotype (homeostasis model assessment [HOMA], IL-6, CRP, soluble IL-2 receptor [IL-2sR], and PAI-1) within each of the four clusters defined by ethnic group. A total of 5,000 permutations were performed for each SNP-phenotype, and the observed statistic was compared with that obtained from the simulations to define the empiric P value. This approach was also used within diabetic/non-diabetic clusters for evaluation of SNP-diabetes interaction. The permutations were performed within PLINK using the max(T) option. This effectively tests the appropriate distributional assumptions of the analyses; should the distributions of the phenotypes deviate significantly from normality, we would expect the permuted P values to be far from those observed. In our case, the permuted P values are consistent with those observed.

RESULTS

Characterization of a novel selective A2BR antagonist ATL-692. Several potent and selective antagonists of the A2BR such as MRS-1754 have been described (17). In general these compounds have poor aqueous solubility, poor bioavailability, and are less potent and selective at rodent than at human A2BRs. We have recently described ATL-801 as a selective A2B blocker with improved water solubility useful for in vivo studies (18). Figure 1 shows the chemical structure and binding characteristics of a new antagonist, ATL-692, with greater potency and selectivity than ATL-801. The synthesis and pharmacological characterization of ATL-692 is described in Supplementary data. In competition for radioligand binding to recombinant adenosine receptors, ATL-692 is >400-fold selective for the A2BR over the other recombinant human, mouse, or rat adenosine receptor subtypes. However, relative to ATL-801, ATL-692 has 10× lower aqueous solubility (3 vs. 30 μg/ml) and 5× lower oral bioavailability in rats (13 vs. 73%). Hence, we used ATL-692 as the preferred compound for in vitro studies, whereas ATL-801 as preferred for in vivo studies.

Effect of A2BR deletion or blockade on glucose metabolism. In previous studies, 2-alkynyl-8-aryladenine adenosine antagonists with selectivity for the A2BR subtype have been reported to have hypoglycemic activity in the KK-A1 mouse model of type 2 diabetes (11). We examined the effects of ATL-801 on insulin sensitivity during

FIG. 1. Structure and adenosine receptor binding characteristics of ATL-692. Kᵢ values for ATL-692 at rat, mouse, and human (h) adenosine receptors are expressed as mean nM ± SE (N = 3) and were calculated from the half-maximal inhibitory concentration (IC₅₀) of ATL-692 to compete for radioligand binding to recombinant receptors on human embryonic kidney (HEK)293 cell membranes. The radioligands used were [125I]-ABA (A₁R and A₂BR), [3H]-ZM241385 (A₃R), and [3H]-ABOPX (A₄R). Binding is plotted as fraction of control specific binding.
hyperinsulinemic-euglycemic clamps in KKA\textsuperscript{Y} mice. Body weight and basal plasma glucose levels were not affected by short-term (2 day) ATL-801 treatment (Fig. 2A and B). During the clamp, plasma glucose levels were maintained at \( \sim 7 \) mmol/L (Fig. 2C). Steady-state rates of glucose infusion to maintain euglycemia during clamps were significantly elevated in ATL-801–treated KKA\textsuperscript{Y} mice as compared with untreated KKA\textsuperscript{Y} controls (Fig. 2D). Insulin-stimulated glucose uptake in skeletal muscle and brown adipose tissue were increased by 20 to \( \sim 50\% \) in KKA\textsuperscript{Y} mice (Fig. 2E and F). Basal and clamp HGP rates were markedly reduced in ATL-801–treated KKA\textsuperscript{Y} mice, resulting in a 30\% increase in hepatic insulin action (Fig. 2G–I). In sum, ATL-801 treatment of diabetic mice increased insulin action in liver and increased glucose uptake in skeletal muscle and brown adipose tissue.

We reasoned that if insulin resistance occurs as a consequence of A\(_{2B}\)R activation, injecting mice with the stable nonselective adenosine receptor agonist 5\'-N-ethylcarboxamidoadenosine (NECA) should activate adenosine receptor–mediated effects and also inhibit glucose disposal. Figure 3A shows that oral gavage with NECA 35 min before an oral glucose tolerance test (GTT) in wild-type fasted mice results in a substantial delay in glucose disposal during GTT. These effects of NECA were somewhat attenuated in mice lacking A\(_1\) or A\(_{2A}\) receptors but were almost completely abolished in mice lacking the A\(_{2B}\)R. Moreover, NECA significantly increased fasting glucose levels in C57BL/6 but not in A\(_{2B}\)R\(^{-/-}\) mice (Fig. 3B). Insulin resistance in response to NECA was associated with an increase in plasma IL-6 measured 4 h after NECA administration (Fig. 3C). Genetic ablation of the A\(_{2B}\)R has
previously been shown to dramatically reduce the ability of NECA to raise IL-6 plasma levels in vivo and to abrogate NECA-induced IL-6 release from mouse peritoneal macrophages (19). The addition of the orally active A2BR antagonist ATL-801 (10 mg/kg/day) to high-fat mouse diet (55% of calories from lipid) for 10 weeks significantly reduced diet-induced elevated fasting blood glucose (Fig. 3D). The results indicate that the effects of adenosine receptor antagonists to reduce insulin resistance are mediated by blockade of the A2BR subtype. In addition, the results suggest that endogenous levels of adenosine in diabetic animals are sufficient to activate A2BRs.

**NECA causes induction of cytokine transcripts in ECs and macrophages.** Because endothelial activation is a hallmark of insulin resistance, we sought to determine whether NECA causes induction of cytokine transcripts in ECs and whether the response is influenced by diabetes. As shown in Fig. 4A and B, NECA triggers a transient increase in IL-6 mRNA and a more sustained stimulation of IL-6 production. NECA also stimulates the production of the murine IL-8 homolog KC (Fig. 4C and D). As shown in Fig. 4E, A2BR mRNA in ECs from diabetic mice (db/db or KKAy) is increased six- to sevenfold compared with ECs derived from age- and sex-matched congenic controls. This induction is associated with a shift to the left in the dose response curve for NECA-induced IL-6 production and an increase in the maximal response (Fig. 4F). To determine whether diabetes causes induction of A2BR mRNA in human tissues, we prepared macrophages from the monocytes of diabetic and nondiabetic individuals. Monocyte populations readily differentiate into macrophages in tissue and, during the differentiation process, retain their genetic identity (20). As shown in Fig. 4G, diabetes is associated with increased expression of A2BR mRNA in macrophages.
derived from human monocytes. We also examined the effect of diabetes on NECA-stimulated IL-6 production in mouse peritoneal macrophages in vitro. As shown in Fig. 4H, diabetes significantly increases A2BR-mediated IL-6 production in mouse macrophages.

**Characterization of adenosine receptors on mouse aortic ECs.** There are differences in the adenosine receptors found on ECs in various vascular beds. To pharmacologically evaluate the adenosine receptor subtype that mediates cytokine release from mouse aortic ECs, we used 100 nmol/L FSPTP, a highly selective A2AR antagonist (22), and 1 μmol/L ATL-692, a highly selective A2BR antagonist (Fig. 1). The A2AR is primarily coupled to Gs, while the A2BR is dually coupled to Gs and to Gq (23). Consistent with Gs coupling, NECA caused a rapid increase in cyclic AMP that is blocked by 1 μmol/L ATL-692 but not affected by FSPTP (Fig. 5A). Thus, although A2ARs are found on some ECs, cyclic AMP accumulation in mouse aortic ECs is exclusively mediated by A2BRs. We used kinase inhibitors to investigate signaling downstream of A2AR activation in ECs. Both Gö6976, an inhibitor of PKC, and KT5720, an inhibitor of PKA, significantly inhibited IL-6 mRNA induction by NECA, and the combination of the two agents had an additive effect (Fig. 5B). Thus both PKA and PKC appear to contribute to induction of IL-6 in response to A2BR activation. In ECs derived from A2BR−/− mice, IL-6 release was reduced to near 0 in the absence or presence of NECA. These findings suggest that constitutive A2AR activity or constitutive production of adenosine by ECs stimulates low-level cytokine production in vitro. This may also occur in vivo where local adenosine production in response to shear stress, platelet activation, or nerve activation likely stimulates endothelial A2BRs and cytokine production. As further confirmation that A2ARs mediate the effects of NECA in mouse aortic ECs, agonists of adenosine receptor subtypes added at doses sufficiently low to exert receptor subtype selectivity (CPA, A1; CGS21680, A2A; and CI-IB-MECA, A3) were found to be without effect on IL-6 production (Fig. 5C).

**SNPs in ADORA2B.** Having established a relationship between diabetes, A2BR mRNA induction, and cytokine production in mice, we examined SNPs in the A2BR gene, ADORA2B, in 2,847 subjects from the MESA for associations between receptor SNPs and diabetes or inflammation. Table 1 lists by SNP genotype the means for HOMA-insulin resistance (HOMA-IR) and inflammatory adipokines, adjusted for covariates. The minor alleles of ADORA2B SNPs (the allele with the lower frequency and thus considered the variant allele) are listed first in the table. For five consecutive tag SNPs numbered 2–5 in the table, there is a striking association of allelic genotype (homozygous minor, heterozygote, homozygous major) with plasma concentrations of IL-6 and CRP. Among the same tag SNPs, the relationship...
is inverted for IL-2sR. These findings indicate that minor allele frequency in ADORA2B SNPs influences the expression of inflammatory markers in the MESA population.

**Effect of diabetes status on association of ADORA2B SNPs with inflammation markers.** In models that included diabetes as an independent predictor of variation in inflammatory markers, the diabetes effect was highly significant ($P < 10^{-15}$) for all ADORA2B SNPs IL-6, CRP, or IL-2sR. We further evaluated the effect of diabetes on the associations between individual ADORA2B SNPs and markers of inflammation. Within patients with diabetes and nondiabetics, clusters were defined to test for homogeneity of SNP association with each phenotype using the Cochran-Mantel-Haenszel approach. Analyses of association between ADORA2B SNPs with individual MESA phenotypes are shown in Table 2, adjusted for covariates (age, sex, ethnicity, site of ascertainment, smoking) and population admixture (first five principal components from ancestry informative markers). Among patients with diabetes, significant associations between one or more SNPs and all four markers of inflammation were noted. In seven instances denoted in the table, there is a $>10$-fold decrease in the $P$ value of SNP associations with inflammatory phenotypes in patients with diabetes compared with nondiabetics.

**DISCUSSION**

IL-6, CRP, and PLA-1 are all adipokines, i.e., proinflammatory mediators produced in adipose tissue, that have been associated with diabetes (24). Inflammation in diabetes may be triggered in part by elevated concentrations of free fatty acids that increase CD11c$^+$ macrophage accumulation and activation in adipose tissue (25). The results of this study suggest that adenosine signaling through the A$_{2B}$R also contributes to insulin resistance by...
TABLE 1
Genotypic means ± SDs of ADORA2B SNPs for MESA phenotypes, combined ethnic groups

| SNP         | Site     | Genotype | HOMA-IR | ln(IL-6) (pg/mL) | ln(CRP) (pg/mL) | ln(IL-2sR) (pg/mL) | PAI-1 (ng/mL) |
|-------------|----------|----------|---------|-----------------|----------------|-------------------|---------------|
| rs7225585 (1)* | 5'       | A/A      | 2.22 (2.69) | 0.15 (0.62)     | 0.68 (0.93)    | -0.33 (0.20)    | 2.73 (0.92)   |
|             |          | G/A      | 2.00 (1.90) | 0.33 (0.66)     | 0.89 (1.17)    | -0.24 (0.39)    | 2.76 (0.84)   |
| rs2779193 (2) | 5'       | A/A      | 2.17 (2.15) | 0.31 (0.65)     | 0.90 (1.02)    | -0.34 (0.37)    | 2.84 (0.87)   |
|             |          | G/A      | 2.06 (2.16) | 0.29 (0.72)     | 0.78 (1.21)    | -0.25 (0.34)    | 2.77 (0.90)   |
| rs758857 (3) | intron 1 | A/A      | 2.02 (2.14) | 0.22 (0.69)     | 0.63 (1.15)    | -0.26 (0.38)    | 2.74 (0.85)   |
| rs8069362 (6) | intron 1 | A/A      | 2.04 (2.03) | 0.30 (0.65)     | 0.89 (1.10)    | -0.27 (0.34)    | 2.87 (0.97)   |
| rs7279211 (9) | intron 1 | A/G      | 1.96 (1.86) | 0.12 (0.70)     | 0.49 (1.15)    | -0.23 (0.28)    | 3.02 (0.96)   |
| rs1045599 (10) | 3'       | A/G      | 1.85 (1.98) | 0.06 (0.70)     | 0.31 (1.18)    | -0.15 (0.37)    | 3.00 (0.81)   |
| rs2286795 (11) | 3'       | A/G      | 1.84 (2.03) | 0.04 (0.69)     | 0.25 (1.16)    | -0.15 (0.38)    | 2.99 (0.83)   |
|             |          | A/A      | 2.00 (2.51) | 0.17 (0.66)     | 0.60 (1.19)    | -0.13 (0.36)    | 2.93 (0.94)   |

Minor (variant) alleles are listed first. The rs7225585 (1) through the rs758857 (3) rows of the HOMA-IR column, the rs2779193 (2) through the rs2041458 (5) rows of the ln(IL-6) column, and the rs7279211 (9) rows of the ln(IL-2sR) column indicate ADORA2B SNPs in which the homozygous minor, heterozygous, and homozygous major alleles are associated with high to low (high, medium, and low plasma inflammatory marker means IL-6, CRP, or PAI-1). In the rs8069362 (6) through rs17715109 (7) rows of the ln(CRP) column, the order of association is reversed, from low to high. *Sequentially numbered tag SNPs referred to in the text. **Small sample size for this allele.

altering the production of IL-6 and other cytokines. IL-6 is produced primarily by macrophages and adipocytes and drives the production of CRP, an acute-phase reactant that increases dramatically during inflammatory processes. We demonstrate six types of associations between diabetes/insulin resistance and A2BRs: 1) diabetes is associated with elevated A2BR mRNA expression in ECs and macrophages, 2) diabetes is associated with elevated A2BR-mediated cytokine production in ECs and macrophages, 3) A2BR activation in mice elevates fasted blood glucose levels, 4) A2BR activation in mice inhibits whole body glucose disposal, 5) A2BR blockade inhibits high-fat diet–induced blood glucose elevation, and 6) A2BR blockade inhibits diabetes-induced insulin-resistance during hyperinsulinemic-euglycemic glucose clamp. Our findings suggest that A2BR blockers may combat insulin resistance by impairing HGP and by attenuating the production of IL-6 and other cytokines that influence glucose and fat metabolism.

Association of ADORA2B SNPs and proinflammatory mediators. SNP analysis seeks to identify significant associations between gene sequences and phenotypes. If a significant association is found, it can then be concluded that the SNP polymorphism, or a nearby polymorphism in a DNA region statistically associated with the SNP, influences either the function or expression of the gene product. Because the current study was not a genome-wide association study, it was not subject to large type I error, i.e., the false apparent associations that can occur when large numbers of genes are analyzed. The genotypic means (minor homozygote, heterozygote, and major homozygote) for each SNP in Table 1 are correlated with increased levels of IL-6 and CRP and decreased levels of IL-2sR. This compelling pattern strongly suggests that one of these SNPs or another SNP in linkage disequilibrium is involved in regulating the function or expression of the A2BR. Our analysis does not enable us to identify which SNP is responsible for altered receptor expression or function. There have been previous attempts to associate particular SNPs in adenosine receptors with diseases. One such study failed to associate coding SNPs in ADORA2B with cystic fibrosis (26). In an investigation of all adenosine receptor genes, a SNP in the 3'UTR of ADORA1 was associated with increased susceptibility to aspirin-intolerant asthma (AIA), whereas another SNP in the coding region was associated with decreased susceptibility. The functional consequences of
ADENOSINE $A_2B$ RECEPTORS AND INSULIN RESISTANCE

TABLE 2
Association of ADORA2B SNPs and inflammatory phenotypes by diabetes status

| SNP        | Site     | ln(IL-6)  | ln(CRP)  | ln(IL-2sR) | PAI-1 |
|------------|----------|-----------|----------|------------|-------|
| rs7225585  | 5’       | 0.042‡ (0.043)* | 0.215 (0.211) | 0.629 (0.627) | 0.333 (0.329) |
| Nondiabetics | 5’       | 0.294 (0.288)† | 0.526 (0.529) | 0.055 (0.056) | 0.315 (0.322) |
| rs758858    | intron 1 | 0.198 (0.189) | 0.189 (0.189) | 0.189 (0.182) | 0.033 (0.037) |
| Patients with diabetes | 0.936 (0.935) | 0.427 (0.428) | 0.535 (0.539) | 0.228 (0.230) |
| rs758858    | 5’       | 0.859 (0.853) | 0.508 (0.502) | 0.043§ (0.050) | 0.153 (0.165)§ |
| Patients with diabetes | 0.284 (0.273) | 0.950 (0.942) | 0.448 (0.449) | 0.0153 (0.014)§ |
| rs2041458   | intron 1 | 0.766 (0.761) | 0.879 (0.876) | 0.185 (0.194) | 0.071 (0.074) |
| Nondiabetics | 0.241 (0.231) | 0.839 (0.836) | 0.051 (0.048) | 0.0445 (0.040) |
| rs8069362   | intron 1 | 0.755 (0.759) | 0.328 (0.333) | 0.317 (0.324§) | 0.252 (0.251) |
| Nondiabetics | 0.079 (0.079) | 0.813 (0.807) | 0.009 (0.010)§ | 0.033 (0.034) |
| rs17715109  | intron 1 | 0.709 (0.714) | 0.945 (0.943) | 0.706 (0.755) | 0.380 (0.377) |
| Nondiabetics | 0.835 (0.843) | 0.230 (0.230) | 0.779 (0.770) | 0.452 (0.455) |
| rs2015353   | intron 1 | 0.463 (0.463)§ | 0.721 (0.716)§ | 0.081 (0.085) | 0.443 (0.466) |
| Patients with diabetes | 0.047§ (0.047)§ | 0.001§ (0.001)§ | 0.154 (0.148) | 0.472 (0.466) |
| rs2779211   | intron 1 | 0.261 (0.253) | 0.469 (0.456)§ | 0.216 (0.221) | 0.731 (0.729) |
| Nondiabetics | 0.112 (0.113) | 0.003§ (0.002§) | 0.825 (0.825) | 0.534 (0.526) |
| rs1045599   | 3’       | 0.499 (0.495) | 0.372 (0.364) | 0.584 (0.590) | 0.763 (0.755) |
| Nonbiabetics | 0.059 (0.058) | 0.050§ (0.049) | 0.496 (0.490) | 0.454 (0.454) |
| rs2286795   | 3’       | 0.303 (0.303) | 0.390 (0.388)§ | 0.936 (0.938) | 0.886 (0.889)§ |
| Patients with diabetes | 0.235 (0.239) | 0.009§ (0.008§) | 0.550 (0.544) | 0.0385 (0.038§) |

*P values from the additive (1 df) model in nondiabetic subjects, adjusted for age, sex, center of ascertainment, pack-years smoking, and ancestry (the first five principal components from 200 AIMs), Bonferroni adjusted (number in parenthesis represents the empiric P value).
†P values from the additive (1 df) model in diabetic subjects, adjusted for age, sex, ethnicity, center of ascertainment, pack-years smoking, and ancestry (the first five principal components from 200 AIMs), Bonferroni adjusted (number in parenthesis represents the empiric P value).
‡P < 0.05. §P value in nondiabetics is >10 × patients with diabetes.

particular variants were not defined. Other SNPs in adenosine deaminase, ADORA2A, ADORA2B, and ADORA3, were not significantly associated with AIA (27). Recently, there has been an explosion of genome-wide and candidate gene association of SNPs with both disease and quantitative (associated) phenotypes. Despite early expectations that SNPs in coding regions of genes would be most significant, most of the SNPs that have been shown to exhibit the strongest associations have been either intronic or intergenic (not in the coding regions). Mutations in these regions are most likely to regulate gene transcription. Hence it is possible that a functional SNP in ADORA2B results in modification of gene transcription. Based on the results of the current study we conclude that in patients with diabetes, signaling through $A_2B$s is influenced by one or more SNPs that modify production of IL-6 and CRP, which in turn influence insulin resistance.

Proinflammatory and anti-inflammatory signaling by $A_2B$s. Deletion of the mouse $A_2B$ resulted in a proinflammatory phenotype manifested as mild vascular inflammation at baseline and exacerbation of cytokine production in response to endotoxin (15). Thus, in some settings, signaling via the $A_2B$s reduces inflammation. On the other hand, in this and several previous studies, activation of $A_2B$s increased IL-6 plasma levels in mice, and by several types of isolated cells (28), including macrophages (19) and dendritic cells (19,29). IL-6 also is directly involved in stimulating the production of transcription factors that enhance CRP production-3, and protein tyrosine phosphatase 1b (32). IL-6 also is directly involved in stimulating the production of transcription factors that enhance CRP production (33). It is interesting that SNP genotypes associated with IL-6 and CRP are inversely associated with another inflammatory marker, IL-2sR (Table 2). Unlike IL-6, CRP, and PAI-1, IL-2sR is not an adipokine and is a
marker of T cell activation. The results suggest that A2BR signaling can result in inhibition of lymphocyte activation, at least in some individuals.

**Adenosine receptor signaling in diabetes.** Previous studies have shown that the stable nonselective adenosine analog NECA stimulates glucose production by hepatocytes (34). In the current study we show that oral gavage with NECA acutely increases fasting glucose levels and strongly inhibits glucose disposal. Both deletion of the A2BR gene and selective A2B blocker with ATL-801 implicate the A2BR as the primary mediator of these responses. These findings indicate that the previously noted effects of adenosine receptor antagonists to reduce diabetes-induced insulin resistance (6–11) can be attributed to adenosine receptor blockade and not to off-target effects. We also observed a small effect of deleting the A1R to increase glucose disposal after NECA administration to mice, possibly due to the known effect of A1R blockade to increase pancreatic insulin secretion (35). Hyperinsulinemic-euglycemic glucose clamps in KKA 

1 mice demonstrate that blockade of A2BR signaling enhances insulin sensitivity and glucose metabolism in skeletal muscle, brown adipose tissue, and liver. These data are consistent with the hypothesis that activation of the A2BR causes insulin resistance that may be mediated in part by cytokine production.

**Association of coffee consumption with diabetes.** The most potent activity of the methylxanthine caffeine is nonselective blockade of A1, A2A, and A2B adenosine receptors (36). It is notable, however, that ATL-692 is about 5,000 times more potent than caffeine as a competitive antagonist of the human A2BR. In human epidemiologic studies, long-term coffee consumption is strongly associated with a reduction in the incidence of type 2 diabetes. However, factors other than caffeine contribute to this effect, and the contribution of caffeine is controversial (37,38). Moreover, blockade of A1Rs acutely counteracts insulin actions by stimulating catecholamine release and by counteracting the antilipolytic effect of A1R activation in adipocytes. Perhaps due to the complex pharmacology of coffee and caffeine, it has not been possible in epidemiologic studies to clearly demonstrate a significant effect of caffeine as a contributor of coffee-induced protection from type 2 diabetes. However, in rigorously controlled studies in diabetic KKA 

1 mice, consumption of high amounts of coffee or equivalent doses of pure caffeine reduce hyperglycemia, decrease fat mass, reduce the expression of tumor necrosis factor-α (TNF-α) and IL-6 in white adipose tissue, and reduce the expression of hepatic genes involved in fatty acid synthesis (39). The results of the current study suggest that at least some of the effects of caffeine in diabetic animals are mediated by blockade of A2BRs. It is pertinent also that in human studies, genetic variability in the activity of polymorphic forms of adenosine deaminase is associated with obesity and type 2 diabetes (40). An increase in the activity of adenosine deaminase, by reducing adenosine levels, has an effect similar to nonselective adenosine receptor blockade produced by caffeine.

**Diabetes and adenosine metabolism.** Human gestational diabetes is associated with elevated extracellular adenosine (41). In rats, diabetes also enhances adenosine accumulation and signaling and diminishes the expression of cytosolic adenosine kinase, the enzyme that converts adenosine to AMP (42). In mice, ablation of the adenosine kinase gene results in severe hepatic steatosis (43) that is strongly associated with diabetes. Hepatic steatosis has been attributed to increased circulating free fatty acids, resulting in liver lipid deposition. Another enzyme involved in adenosine production is the ecto-5'-nucleotidase CD73, which converts AMP to adenosine in the extracellular space. It is notable that statins stimulate the induction of CD73 and have been shown in numerous studies to elicit insulin resistance. Statins also enhance ischemia-mediated vasodilation in humans that is blocked by caffeine, consistent with an effect to enhance adenosine production (44). We speculate that enhanced adenosine production, by activating A2BRs, may contribute to the well-known effect of statins to provoke insulin resistance (45).

**Diabetes and regulation of A2BR transcription.** In the current study we demonstrate that diabetes triggers induction of A2AR mRNA in macrophages and ECs, resulting in increased cytokine production in response to A2BR activation. Analyses of the cloned human A2R promoter identified a functional binding site for hypoxia-inducible factor (46) and identified TNF-α and the oxidative stress-promoting enzyme NAD(P)H oxidase as additional regulators of A2BR gene expression (47). Because elevated TNF-α and oxidative stress are associated with diabetes (48,49), it is reasonable to speculate that these factors contribute to induction of A2BR mRNA in patients with diabetes. We noticed a strong effect of diabetes on the probability of associations between ADORA2B SNPs and inflammatory markers (Table 2). A2BR signaling in nondiabetics due to low adenosine levels and low A2BR expression could render SNPs in ADORA2B that might influence A2BR signaling inconsequential in this population. In patients with diabetes, on the other hand, strong A2BR signaling may enhance functional consequences of ADORA2B SNPs. The findings of this study, in particular the induction of A2BR mRNA in ECs and macrophages from diabetic animals, are consistent with the possibility that one or more SNPs in ADORA2B influences A2BR mRNA expression. It will be of interest in future studies to determine whether ADORA2B genotypes are associated with A2BR mRNA expression in human monocytes.

In sum, the results of this study are consistent with the idea that diabetes enhances signaling through A2BRs both by elevating adenosine levels and by increasing the expression of the A2BR. Our findings indicate that A2BR signaling can facilitate the release of proinflammatory cytokines from human macrophages and mouse ECs. Blockade or deletion of the A2BR reverses the effects of diabetes on cytokine production and insulin resistance assessed by GTT or hyperinsulinemic-euglycemic clamp. The minor (variant) allele of several (sequential) tag SNPs in ADORA2B is strongly correlated with IL-6 and CRP, acute phase proteins that are highly associated with diabetes. We also observed a strong effect of diabetes on the association between ADORA2B SNPs and markers of inflammation. These findings suggest that both diabetes and ADORA2B genotype can influence A2BR expression. It will be of interest to determine whether new potent and selective A2BR blockers that are currently in clinical development are effective in reducing obesity or insulin resistance in human disease.

**ACKNOWLEDGMENTS**

This work was supported by NIH Grants R01-HL-37942 (to J.L.) and R01-DK-80756 (to J.K.K.) and by MESA contracts N01-HC-95159 through N01-HC-95165 and N01-HC-95169 from the National Heart, Lung, and Blood Institute. Additional
support to J.K.K. was provided by the American Diabetes Association, 7-07-RA, and the Pennsylvania State Department of Health Tobacco Settlement Award.

No potential conflicts of interest relevant to this article were reported.

R.A.F. did experiments on ECs and macrophages. G.W. synthesized ATL-692. S.S. did experiments on ECs and macrophages. K.F.L. designed GTTs. D.Y.J. and Z.Z. executed and analyzed euglycemic clamp studies. J.S.P. executed and analyzed euglycemic clamp studies. J.L. provided experimental SNP analysis and interpretation. J.K.K. executed and analyzed euglycemic clamp studies. J.S.P. executed and analyzed euglycemic clamp studies. J.L. provided experimental design and wrote the article. S.S.R. executed and analyzed euglycemic clamp studies. J.L. provided experimental design and wrote the article.

The authors thank Jiang-Fan Chen of Boston University for \( \Delta \alpha_{\text{A2A}} \) \(-/\)-mice. The authors also thank the other investigators, the staff, and the participants of the MESA study for their valuable contributions. A full list of participating MESA investigators and institutions can be found at http://www.mesa-nhlbi.org.

REFERENCES

1. Bastard JP, Jardel C, Bruckert E, et al. Elevated levels of interleukin 6 are reduced in serum and subcutaneous adipose tissue of obese women after weight loss. J Clin Endocrinol Metab 2000;85:3338–3342
2. Kahn SE, Zinman B, Haffner SM, et al.; ADOPt Study Group. Obesity is a major determinant of the association of C-reactive protein levels and the metabolic syndrome in type 2 diabetes. Diabetes 2006;55:2357–2364
3. Ma L, Mao SL, Taylor KL, et al. Prevention of obesity and insulin resistance in mice lacking plasminogen activator inhibitor 1. Diabetes 2004; 53:336–346
4. Shaul ME, Bennett G, Strissel KJ, Greenberg AS, Ohin MS. Dynamic, M2-like remodeling phenotypes of CD11c+ adipose tissue macrophages during high-fat diet–induced obesity in mice. Diabetes 2010;59:1171–1181
5. Goldberg RB. Cytokine and cytokine-like inflammation markers, endothelial dysfunction, and imbalanced coagulation in development of diabetes and its complications. J Clin Endocrinol Metab 2009;94:3171–3182
6. Challis RA, Budohoski L, McManus B, Newsholme EA. Effects of an adenosine-receptor antagonist on insulin-resistance in soleus muscle from obese Zucker rats. Biochem J 1984;221:915–917
7. Budohoski L, Challis RA, Cooney GJ, McManus B, Newsholme EA. Reversal of dietary-induced insulin resistance in muscle of the rat by adenosine deaminase and an adenosine-receptor antagonist. Biochem J 1984; 224:327–330
8. Xu B, Berchik DA, Crist GH, LaNoe KF. AI adenosine receptor antagonism improves glucose tolerance in Zucker rats. Am J Physiol 1998;274: E271–E270
9. Crist GH, Xu B, Berchik DA, LaNoe KF. Effects of adenosine receptor antagonism on protein tyrosine phosphorylation in rat skeletal muscle. Int J Biochem Cell Biol 2001;33:817–830
10. Crist GH, Xu B, LaNoe KF, Lang CH. Tissue-specific effects of in vivo adenosine receptor blockade on glucose uptake in Zucker rats. FASEB J 1998;12:1301–1308
11. Harada H, Asano O, Hoshino Y, et al. 2-Alkynyl-8-aryladenines possessing an amide moiety: their synthesis and structure-activity relationships of effects on hepatic glucose production induced via agonism of the A(2B) adenosine receptor. Bioorg Med Chem Lett 2007;17:4503–4506
12. Treadway JL, Saccia R, Jones BK. Adenosine A2B receptor knock-out mice display an improved metabolic phenotype (Abstract). Diabetologia 2006;49:44–45
13. Olsson T, Cronberg T, Ryttet A, et al. Deletion of the adenosine A1 receptor gene does not alter neuronal damage following ischaemia in vivo or in vitro. Eur J Neurosci 2004;20:1197–1204
14. Day JD, Marshall MA, Huang L, McHugh MJ, Okusa MD, Linden J. Protection from ischemic liver injury by activation of A2A adenosine receptors during reperfusion: inhibition of chemokine induction. Am J Physiol Gastrointest Liver Physiol 2004;286:G285–G290
15. Yang D, Zhang Y, Nguyen HG, et al. The A2B adenosine receptor protects against inflammation and excessive vascular adhesion. J Clin Invest 2006;116:1913–1923
16. Bittl JB, Blumer DA, Burke GL, et al. Multi-Ethnic Study of Atherosclerosis: Objectives and design. Am J Epidemiol 2002;156:871–881
17. Kim YC, Ji X, Melman N, Linden J, Jacobson KA. Anilide derivatives of an 8-phenylxanthine carboxylic congener are highly potent and selective antagonists at human A(2B) adenosine receptors. J Med Chem 2000;43:1165–1172
18. Kolachala V, Ruble B, Vijay-Kumar M, et al. Blockade of adenosine A2B receptors ameliorates murine colitis. Br J Pharmacol 2008;155:127–137
19. Ryzhov S, Zaynagetdinov R, Goldstein AE, et al. Effect of A2B adenosine receptor gene ablation on adenosine-dependent regulation of proinflammatory cytokines. J Pharmacol Exp Ther 2008;324:694–700
20. Tacke F, Randolph GJ. Migratory fate and differentiation of blood monocyte subsets. Immunobiology 2006;211:600–618
21. Randolph GJ. The fate of monocytes in atherosclerosis. J Thromb Haemost 2009;7(Suppl. 1):28–30
22. Shroyck JC, Snowdy S, Baraldi PG, et al. A2A-adenosine receptor reserve for coronary vasodilation. Circulation 1998;98:711–718
23. Linden J, Thai T, Figler H, Jin X, Roveba AS. Characterization of human A(2B) adenosine receptors: radioligand binding, western blotting, and coupling to G(q) in human embryonic kidney 293 cells and HMC-1 mast cells. Mol Pharmacol 2008;76:705–713
24. Lau DC, Dhillon B, Yan H, Szmitko PE, Verma S. Adipokines: molecular links between obesity and atherosclerosis. Am J Physiol Heart Circ Physiol 2005;288:H2031–H2041
25. Nguyen MT, Faveluyis K, Nguyen AK, et al. A subpopulation of macrophages infiltrates hypertrophic adipose tissue and is activated by free fatty acids via Toll-like receptors 2 and 4 and JNK-dependent pathways. J Biol Chem 2007;282:35279–35292
26. Tang CM, Hoering A, Bascher R, et al. Human adenosine 2B receptor: SNP discovery and evaluation of expression in patients with cystic fibrosis. Pharmacogenet Genomics 2005;15:321–327
27. Kim SH, Kim YK, Park HW, et al. Adenosine deaminase and adenosine receptor polymorphisms in aspirin-intolerant asthma. Respir Med 2009;103:356–363
28. Linden J. New insights into the regulation of inflammation by adenosine. J Clin Invest 2006;116:1835–1837
29. Novitskiy SV, Ryzhov S, Zaynagetdinov R, et al. Adenosine receptors in regulation of dendritic cell differentiation and function. Blood 2008;112:1822–1831
30. Wallenius V, Wallenius K, Ahren B, et al. Interleukin-6-deficient mice develop mature-onset obesity. Nat Med 2002;8:75–79
31. Fève B, Bastard JP. The role of interleukins in insulin resistance and type 2 diabetes mellitus. Nat Rev Endocrinol 2009;5:305–311
32. Nieto-Vazquez I, Fernández-Veleido S, de Alvaro C, Lorenzo M. Dual role of interleukin-6 in regulating insulin sensitivity in murine skeletal muscle. Diabetes 2008;57:3211–3221
33. Young DP, Kushner I, Sanois D. Binding of C/EBPbeta to the C-reactive protein (CRP) promoter in HepG2B cells is associated with transcription of CRP mRNA. J Immunol 2008;181:2420–2427
34. Harada H, Asano O, Kawata T, et al. 2-Alkynyl-8-aryladenines possessing an amide moiety: their synthesis and structure-activity relationships of effects on hepatic glucose production induced via agonism of the A(2B) adenosine receptor. Bioorg Med Chem Lett 2001;9:2709–2716
35. Johansson SM, Salehi A, Sandstrom ME, et al. AI receptor deficiency causes increased insulin and glucagon secretion in mice. Biochem Pharmacol 2007;74:1628–1635
36. Beukers MW, Meurs I, Ijzerman AP. Structure-affinity relationships of adenosine A2B receptor ligands. Med Res Rev 2006;26:667–698
37. van Dieren S, Uitterwaal CS, van der Schouw YT, et al. Coffee and tea consumption and risk of type 2 diabetes. Diabetologia 2009;52:2561–2569
38. Salazar-Martinez E, Willett WC, Ascherio A, et al. Coffee consumption and risk for type 2 diabetes mellitus. Ann Intern Med 2004;141:1–8
39. Yamachii R, Kobayashi M, Matsuura Y, et al. Coffee and caffeine ameliorate hyperglycemia, fatty liver, and inflammatory adipocytokine expression in spontaneously diabetic KK-Ay mice. J Agric Food Chem 2010;58:5597–5603
40. Bottini E, Gloria-Bottini F. Adenosine deaminase and body mass index in non-insulin-dependent diabetes mellitus. Metabolism 1999;48:949–951
41. San Martin R, Sorelias L, Gestational diabetes and the adenosine/L-arginine/nitric oxide (ALANO) pathway in human umbilical vein endothelium. Planta Med 2006;27:1–10
42. Sakowicz-Burkiewicz M, Kocbuch K, Griden M, Szutowicz A, Pawelczyk T. Diabetes-induced decrease of adenosine kinase expression impairs the proliferation potential of diabetic rat T lymphocytes. Immunology 2006;118:402–412
43. Boison D, Scheurer L, Zumsteg V, et al. Neonatal hepatic steatosis by disruption of the adenosine kinase gene. Proc Natl Acad Sci USA 2002;99: 6985–6990

44. Meijer P, Wouters CW, van den Broek PH, et al. Upregulation of ecto-5’-nucleotidase by rosuvastatin increases the vasodilator response to ischemia. Hypertension 2010;56:722–727

45. Lalli CA, Pauli JR, Prada PO, et al. Statin modulates insulin signaling and insulin resistance in liver and muscle of rats fed a high-fat diet. Metabolism 2008;57:57–65

46. Kong T, Westerman KA, Faigle M, Eltzschig HK, Colgan SP. HIF-dependent induction of adenosine A2B receptor in hypoxia. FASEB J 2006;20:2242–2250

47. Kolachala V, Asamoah V, Wang L, et al. TNF-alpha upregulates adenosine 2b (A2b) receptor expression and signaling in intestinal epithelial cells: a basis for A2bR overexpression in colitis. Cell Mol Life Sci 2005;62:2647–2657

48. Gokulakrishnan K, Mohanavalli KT, Monickaraj F, Mohan V, Balasubramanyam M. Subclinical inflammation/oxidation as revealed by altered gene expression profiles in subjects with impaired glucose tolerance and type 2 diabetes patients. Mol Cell Biochem 2009;324:173–181

49. Castoldi G, Galimberti S, Riva C, et al. Association between serum values of C-reactive protein and cytokine production in whole blood of patients with type 2 diabetes. Clin Sci (Lond) 2007;113:103–108