Links Between Insulin Resistance, Adenosine A_{2B} Receptors, and Inflammatory Markers in Mice and Humans

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OBJECTIVE—To determine the mechanisms by which blockade of adenosine A_{2B} receptors (A_{2B}Rs) reduces insulin resistance.

RESEARCH DESIGN AND METHODS—We investigated the effects of deleting or blocking the A_{2B}R on insulin sensitivity using glucose tolerance tests (GTTs) and hyperinsulinemic-euglycemic clamps in mouse models of type 2 diabetes. The effects of diabetes on A_{2B}R 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 A_{2B}R gene, *ADORA2B*, were evaluated for associations with markers of diabetes and inflammation.

RESULTS—Treatment of mice with the nonselective adenosine receptor agonist 5′-*N*-ethylcarboxamidoadensoine (NECA) increased fasting blood glucose and slowed glucose disposal during GTTs. These responses were inhibited by A_{2B}R deletion or blockade and minimally affected by deletion of A_1Rs or A_{2A}Rs. During hyperinsulinemic-euglycemic clamp of diabetic KKA^y mice, A_{2B}R 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 A_{2B}R 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 A_{2B}Rs that stimulate IL-6 and CRP production, insulin resistance, and the association between *ADORA2B* SNPs and inflammatory markers. We hypothesize that increased A_{2B}R signaling in diabetes increases insulin resistance in part by elevating proinflammatory mediators. Selective A_{2B}R blockers may be useful to treat insulin resistance. ***Diabetes*** **60:669–679, 2011**

Obesity 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 NH_2-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 A_1/A_{2B} orally active antagonist, BW-1433, was found to persistently 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 A_1Rs. This idea was corrected when moderately selective blockers of the A_{2B}R were found to lower glucose levels in diabetic mice, an effect that could not be replicated with the selective A_1R 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 A_{2B}R 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 A_{2B}Rs 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 A_{2B}R signaling.

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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′-cttgaaggtgttgccctcag-3′/5′-tggggacacctttagcatc-3′; IL-6 5′-ctgatgctggtgacaaccac-3′/5′-tccacgatttcccagagaac-3′; A_{2A}R 5′-tggcttggtgacgggtatg-3′/5′-cgcaggtctttgtggagttc-3′; and A_{2B}R 5′-ctgggacacgagcgagag-3′/5′-gctggtggcactgtctttac-3′. Sense/antisense human PCR primers were A_{2A}R 5′-agttccgccagaccttcc-3′/5′-acctgctctccgtcactg-3′; A_{2B}R 5′-ggtcattgctgtcctctg-3′/5′-ttcattcgtggttccatcc-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, Tukwila, 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 A_{2A}R, A_{2B}R, 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 congenic to C57BL/6 and were created as described previously: $A_1R^{-/-}$ (13), $A_{2A}R^{-/-}$ (14), and $A_{2B}R^{-/-}$ (15). Some studies used diabetic B6.Cg-$m^{+/+}$Lepr^{db}/J (db/db) with nondiabetic C57BL/6J controls or diabetic KK.Cg-A^y/J (KK-A^y) with less diabetic KK/^{l}H J (KK-a/a) controls as identified in the figure legends. Feeding a high-fat diet (55% calories from fat, Harlan TD93075 ± 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 hyperinsulinemic-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 internal 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 hyperinsulinemic-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 [^{3}H]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 [^{3}H]glucose, ^3H_2O, 2-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^y 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 2,880 MESA subjects (720 in 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 phenotypting 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). Empiric *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/nondiabetic 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 A_{2B}R antagonist ATL-692. Several potent and selective antagonists of the A_{2B}R 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 A_{2B}Rs. We have recently described ATL-801 as a selective A_{2B} 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 A_{2B}R 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 A_{2B}R deletion or blockade on glucose metabolism. In previous studies, 2-alkynyl-8-aryladenine adenosine antagonists with selectivity for the A_{2B}R subtype have been reported to have hypoglycemic activity in the KK-A^Y mouse model of type 2 diabetes (11). We examined the effects of ATL-801 on insulin sensitivity during

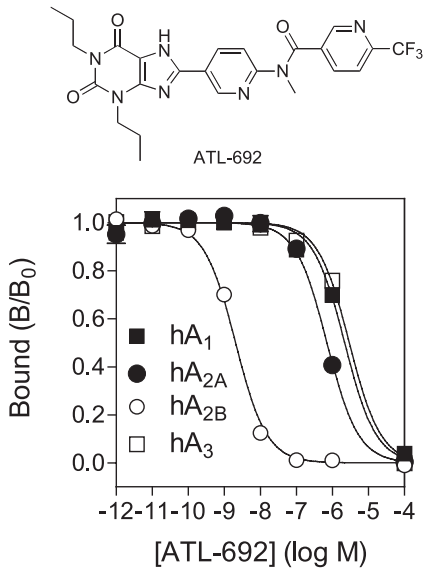

FIG. 1. Structure and adenosine receptor binding characteristics of ATL-692. K_i values for ATL-692 at rat, mouse, and human (h) adenosine receptors are expressed as mean nM ± SE (N = 3) and were calculated from the half-maximal inhibitory concentration (IC_{50}) of ATL-692 to compete for radioligand binding to recombinant receptors on human embryonic kidney (HEK)293 cell membranes. The radioligands used were ^{125}I-ABA (A_1R and A_3R), ^{125}I-ZM241385 (A_{2A}R), and ^{125}I-ABOPX (A_{2B}R). Binding is plotted as fraction of control specific binding.

hyperinsulinemic-euglycemic clamps in KKA^Y mice. Body weight and basal plasma glucose levels were not affected by short-term (2 day) ATL-801 treatment (Fig. 2*A* and *B*). During the clamp, plasma glucose levels were maintained at ~7 mmol/L (Fig. 2*C*). Steady-state rates of glucose infusion to maintain euglycemia during clamps were significantly elevated in ATL-801–treated KKA^Y mice as compared with untreated KKA^Y controls (Fig. 2*D*). Insulin-stimulated glucose uptake in skeletal muscle and brown adipose tissue were increased by 20 to ~50% in KKA^Y mice (Fig. 2*E* and *F*). Basal and clamp HGP rates were markedly reduced in ATL-801–treated KKA^Y mice, resulting in a 30% increase in hepatic insulin action (Fig. 2*G–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*-ethylcarboxamidoadensoine (NECA) should activate adenosine receptor–mediated effects and also inhibit glucose disposal. Figure 3*A* 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. 3*B*). Insulin resistance in response to NECA was associated with an increase in plasma IL-6 measured 4 h after NECA administration (Fig. 3*C*). Genetic ablation of the $A_{2B}R$ has

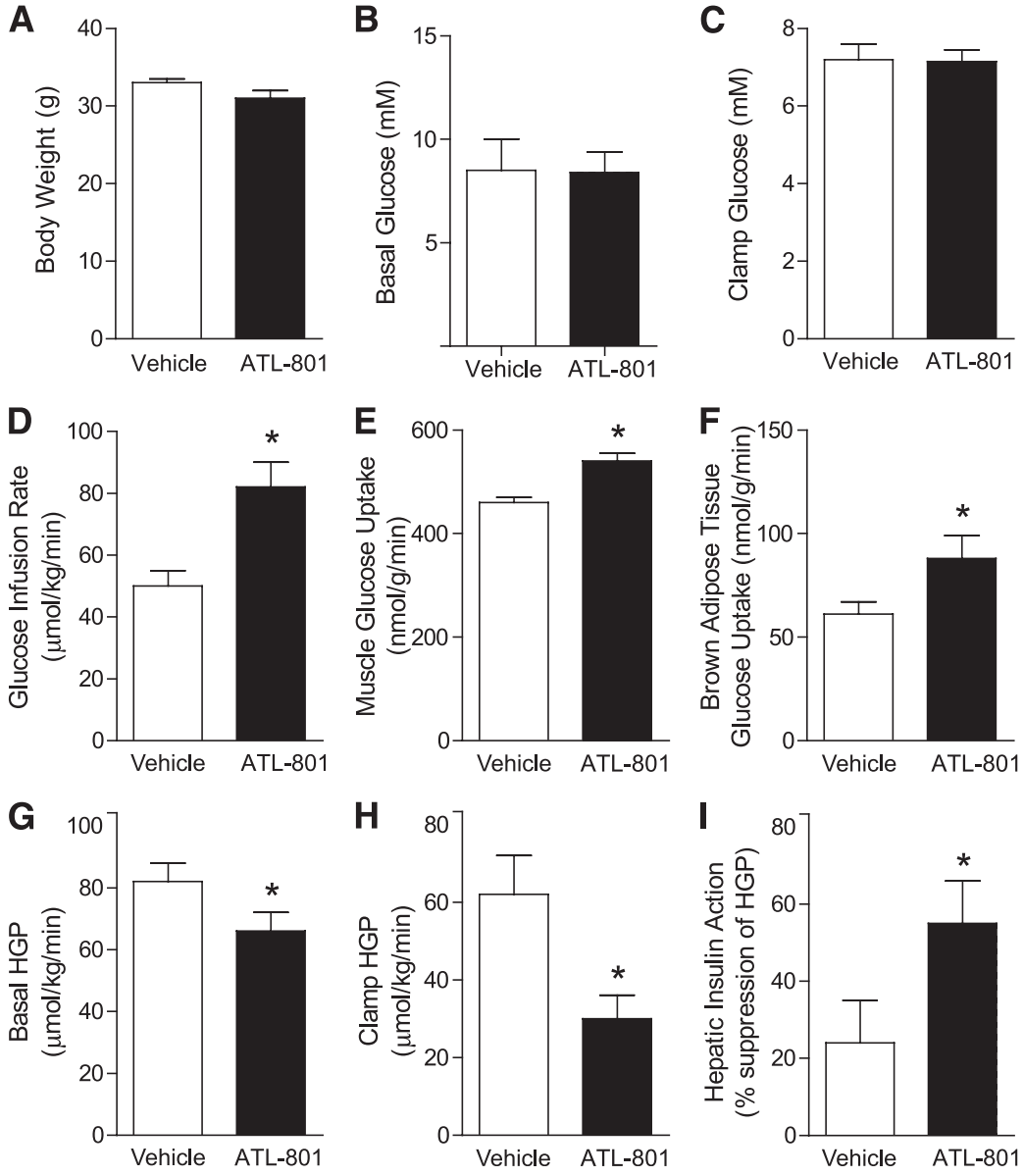

FIG. 2. ATL-801 treatment increases insulin sensitivity in KKA^Y mice. ATL-801 was administered by oral gavage 4 times at 12-h internals with the last dose given 4 h before clamp. *A*: Body weight change during ATL-801 administration. *B*: Basal plasma glucose levels. *C*: Plasma glucose levels during clamps. *D*: Steady-state glucose infusion rates during clamps. *E*: Insulin-stimulated skeletal muscle glucose uptake. *F*: Insulin-stimulated glucose uptake in brown adipose tissue. *G*: Basal HGP. *H*: HGP during clamps (insulin-stimulated state). *I*: Hepatic insulin action reflected as insulin-mediated percent suppression of basal HGP. *$P < 0.05$ by two-tailed Student *t* test; $N = 7$.

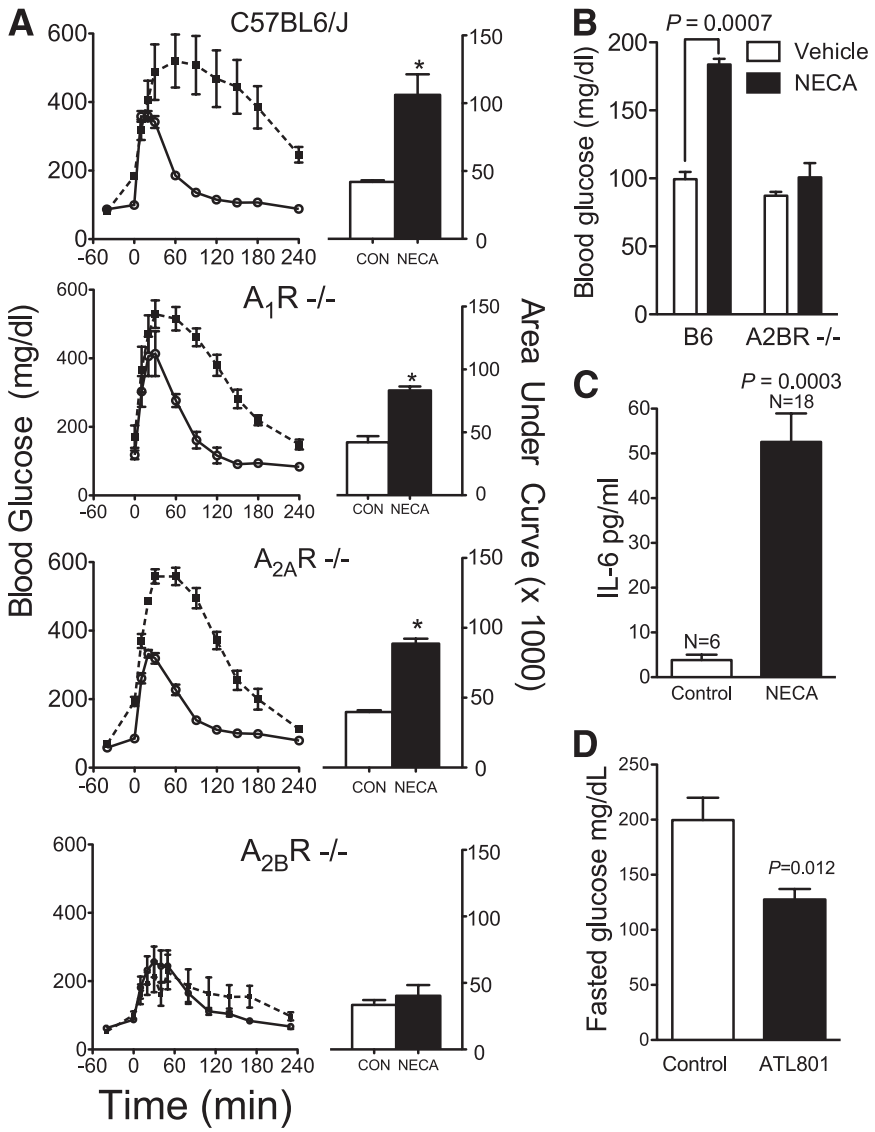

FIG. 3. A_{2B}R-mediated regulation of glucose metabolism. *A*: C57BL/6J and adenosine receptor knockout mice (male, 8 weeks, *N* = 5) received an oral bolus of vehicle or NECA (0.3 mg/kg) at time −35. At time 0, mice were subjected to an GTT (1.25 g/kg ip). At the indicated time points, blood glucose levels were determined using a OneTouch Ultra glucometer (LifeScan, Milpitas, CA) and area under the curve (AUC) was calculated using GraphPad PRISM software. *$P < 0.0001$. *B*: Fasting glucose levels were measured in wild-type and $A_{2B}R^{-/-}$ mice 35 min after NECA gavage (*N* = 5). *C*: Plasma IL-6 was measured 4 h after C57BL/6J mice received vehicle or NECA. *D*: C57BL/6 mice were fed a high-fat diet ± 10 mg/kg/day of ATL-801 for 10 weeks. Blood glucose was measured after a 4-h fast (*N* = 5). Con, control.

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 A_{2B}R 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. 3*D*). The results indicate that the effects of adenosine receptor antagonists to reduce insulin resistance are mediated by blockade of the A_{2B}R subtype. In addition, the results suggest that endogenous levels of adenosine in diabetic animals are sufficient to activate A_{2B}Rs.

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. 4*A* 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. 4*C* and *D*). As shown in Fig. 4*E*, A_{2B}R mRNA in ECs from diabetic mice (db/db or KKA^{Y}) 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. 4*F*). To determine whether diabetes causes induction of A_{2B}R 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). These macrophages, and not their monocyte precursors, are the culprits in inflammation and disease (21). As shown in Fig. 4*G*, diabetes is associated with increased expression of A_{2B}R mRNA in macrophages

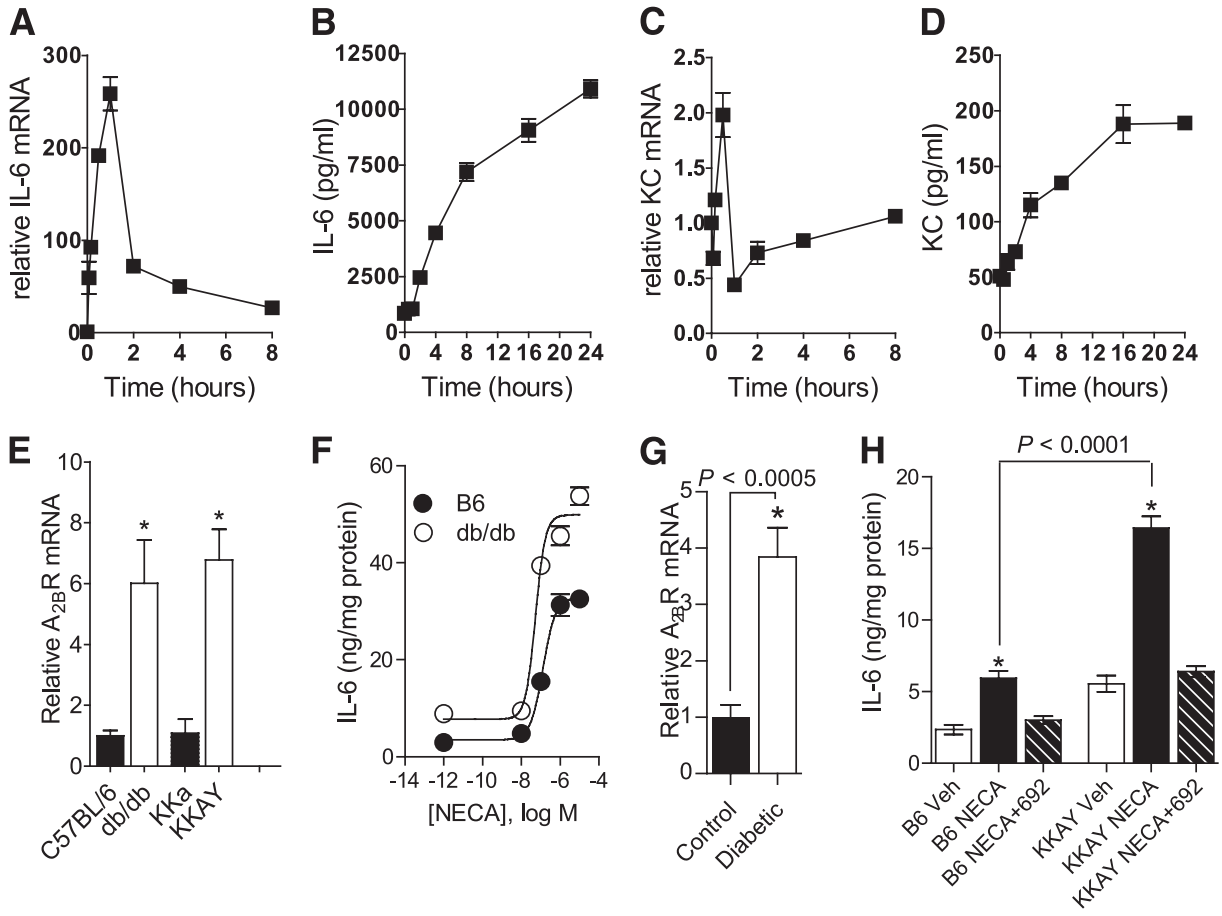

FIG. 4. $A_{2B}R$ transcription and function in ECs and macrophages. Time courses of responses to 1 μmol/L NECA in mouse aortic ECs are IL-6 mRNA (*A*), IL-6 protein (*B*), KC mRNA (*C*), and KC protein (*D*). *E*: Effect of diabetes in two strains of mice on EC $A_{2B}R$ mRNA relative to congenic nondiabetic controls. *$P < 0.05$. *F*: Effect of diabetes on the dose-response curve of NECA to stimulate IL-6 production in ECs at 24 h. The concentrations of NECA that produce 50% of maximal responses (EC_{50}) are 140 nmol/L C57BL/6 and 46 nmol/L db/db. *G*: $A_{2B}R$ mRNA in monocyte-derived macrophages prepared from controls or diabetic human donors ($N = 6$). *H*: IL-6 production in C57BL/6 ($N = 3$) or KKA^{Y} ($N = 6$) mouse peritoneal macrophages treated in vitro for 24 h with vehicle (Veh), 1 μmol/L NECA, or NECA + 1 μmol/L ATL-692. *$P < 0.01$.

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. 4*H*, diabetes significantly increases $A_{2B}R$-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 $A_{2A}R$ antagonist (22), and 1 μmol/L ATL-692, a highly selective $A_{2B}R$ antagonist (Fig. 1). The $A_{2A}R$ is primarily coupled to G_s, while the $A_{2B}R$ is dually coupled to G_s and to G_q (23). Consistent with G_s coupling, NECA caused a rapid increase in cyclic AMP that is blocked by 1 μmol/L ATL-692 but not affected by FSPTP (Fig. 5*A*). Thus, although $A_{2A}Rs$ are found on some ECs, cyclic AMP accumulation in mouse aortic ECs is exclusively mediated by $A_{2B}Rs$. We used kinase inhibitors to investigate signaling downstream of $A_{2B}R$ 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. 5*B*). Thus both PKA and PKC appear to contribute to induction of IL-6 in response to $A_{2B}R$ activation. In ECs derived from $A_{2B}R^{-/-}$ mice, IL-6 release was reduced to near 0 in the absence or presence of NECA. These findings suggest that constitutive $A_{2B}R$ 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 $A_{2B}Rs$ and cytokine production. As further confirmation that $A_{2B}Rs$ mediate the effects of NECA in mouse aortic ECs, agonists of adenosine receptors subtypes added at doses sufficiently low to exert receptor subtype selectivity (CPA, A_1; CGS21680, A_{2A}; and Cl-IB-MECA, A_3) were found to be without effect on IL-6 production (Fig. 5*C*).

SNPs in ADORA2B. Having established a relationship between diabetes, $A_{2B}R$ mRNA induction, and cytokine production in mice, we examined SNPs in the $A_{2B}R$ 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

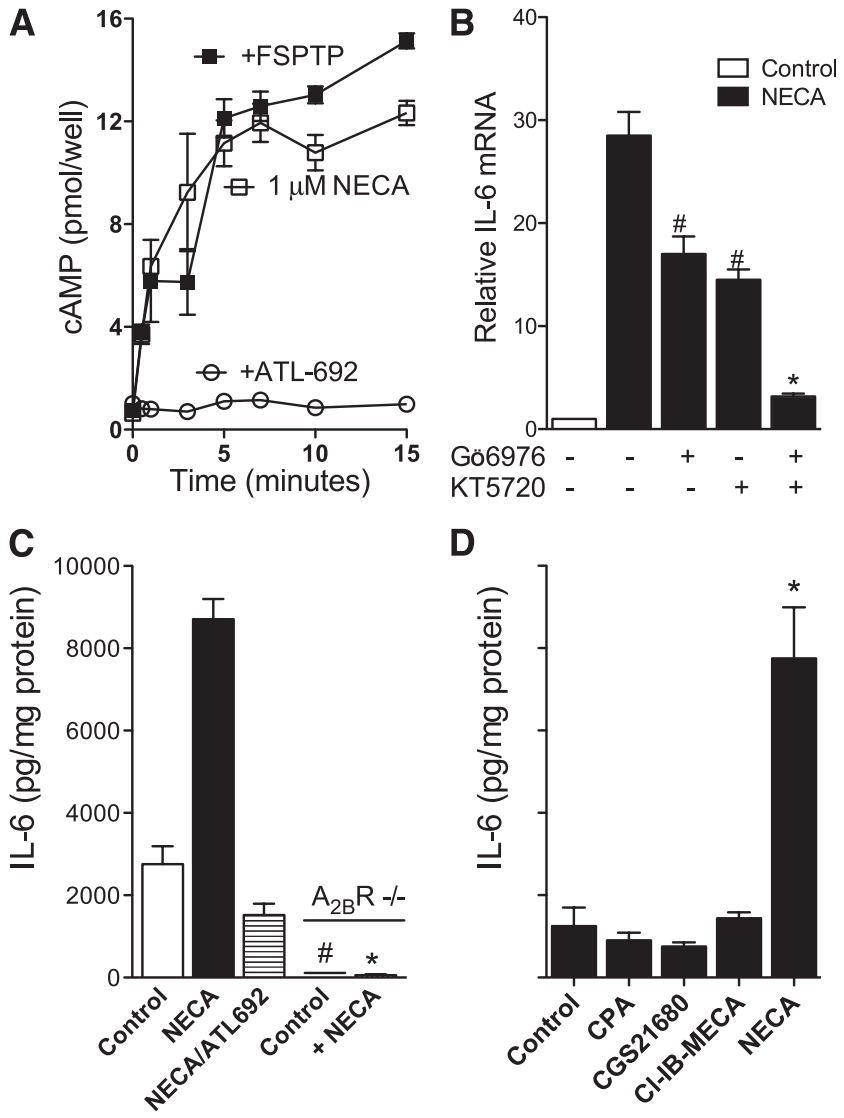

FIG. 5. NECA increases IL-6 production in mouse aortic ECs by activating A_{2B}Rs. *A*: Time course of NECA to increase cyclic AMP in ECs from C57BL/6 mice, and the effects of antagonists, 100 nmol/L FSPTP, or 1 μmol/L ATL-692 (N = 6). *B*: Induction of IL-6 mRNA by NECA in ECs is attenuated by inhibitors of PKC (1 μmol/L Gö6976) or PKA (1 μmol/L KT5720). #$P < 0.05$ vs. no inhibitors. The combination of inhibitors is more effective than either alone (N = 6, *$P < 0.01$). *C*: ECs from C57BL/6J and $A_{2B}R^{-/-}$ mice were stimulated for 16 h with NECA (100 nmol/L) ± ATL-692. IL-6 production in the $A_{2B}R^{-/-}$ ECs is significantly lower than basal C57BL/6J ECs (#$P < 0.001$). *D*: ECs were treated for 16 h with 10 nmol/L CPA (A_1R agonist), 100 nmol/L CGS21680 (A_{2A}R agonist), 10 nmol/L Cl-IBMECA (A_3R agonist), or 100 nmol/L NECA (nonspecific agonist). Media were collected and assayed for IL-6 by ELISA (N = 6). *$P < 0.01$ vs. other treatments.

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 ($P \approx 10^{-17}$) 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 Cochrane-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 PIA-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	5′	A/A	2.22 (2.69)	0.15 (0.62)	0.68 (0.93)	−0.33 (0.20)	2.73 (0.92)
(1)*		G/A	2.00 (1.90)	0.33 (0.66)	0.89 (1.17)	−0.24 (0.39)	2.76 (0.84)
		G/G	1.89 (2.09)	0.15 (0.68)	0.52 (1.18)	−0.12 (0.37)	2.93 (0.92)
rs2779193	5′	A/A	2.17 (2.15)	0.31 (0.65)	0.90 (1.02)	−0.34 (0.37)	2.84 (0.87)
(2)		G/A	2.06 (2.16)	0.29 (0.72)	0.78 (1.21)	−0.25 (0.34)	2.77 (0.90)
		G/G	1.87 (2.07)	0.14 (0.66)	0.52 (1.17)	−0.11 (0.37)	2.93 (0.91)
rs758857	intron 1	A/A	2.02 (2.14)	0.22 (0.69)	0.63 (1.15)	−0.26 (0.38)	2.74 (0.85)
(3)		G/A	1.97 (2.05)	0.17 (0.69)	0.59 (1.22)	−0.16 (0.36)	2.92 (0.91)
		G/G	1.82 (2.11)	0.15 (0.66)	0.53 (1.16)	−0.09 (0.37)	2.94 (0.92)
rs758858	intron 1	A/A	1.95 (1.33)	0.36 (0.68)	0.88 (1.17)	−0.34 (0.32)	2.69 (0.81)
(4)		G/A	2.02 (2.08)	0.27 (0.71)	0.77 (1.18)	−0.26 (0.35)	2.72 (0.88)
		G/G	1.89 (2.11)	0.14 (0.66)	0.52 (1.18)	−0.11 (0.37)	2.94 (0.91)
rs2041458	intron 1	A/A	1.94 (1.86)	0.31 (0.66)	0.86 (1.11)	−0.32 (0.32)	2.68 (0.86)
(5)		C/A	2.08 (2.14)	0.28 (0.69)	0.80 (1.17)	−0.22 (0.35)	2.78 (0.88)
		C/C	1.87 (2.10)	0.13 (0.67)	0.48 (1.18)	−0.11 (0.38)	2.95 (0.92)
rs8069362	intron 1	A/A	1.90 (1.72)	0.30 (0.67)	0.94 (1.14)	−0.33 (0.37)	2.52 (0.80)
(6)		G/A	2.04 (2.03)	0.30 (0.65)	0.89 (1.10)	−0.27 (0.34)	2.79 (0.87)
		G/G	1.89 (2.11)	0.14 (0.68)	0.51 (1.19)	−0.12 (0.37)	2.93 (0.92)
rs17715109	intron 1	A/A	1.45 (0.90)	−0.08 (0.40)	−0.14 (1.03)	−0.30**	2.30**
(7)		C/A	1.96 (1.86)	0.12 (0.70)	0.49 (1.15)	−0.23 (0.28)	3.02 (0.96)
		C/C	1.91 (2.12)	0.18 (0.67)	0.59 (1.19)	−0.13 (0.38)	2.89 (0.90)
rs2015353	intron 1	G/G	1.86 (2.91)	0.21 (0.64)	0.73 (1.11)	−0.05 (0.41)	2.85 (0.95)
(8)		A/G	1.97 (1.77)	0.23 (0.66)	0.69 (1.19)	−0.14 (0.35)	2.86 (0.92)
		A/A	1.88 (2.00)	0.09 (0.70)	0.40 (1.18)	−0.20 (0.36)	3.00 (0.86)
rs2779211	intron 1	G/G	1.86 (3.11)	0.19 (0.65)	0.71 (1.12)	−0.02 (0.41)	2.88 (0.97)
(9)		A/G	1.96 (1.78)	0.23 (0.66)	0.67 (1.19)	−0.12 (0.36)	2.86 (0.92)
		A/A	1.89 (1.95)	0.12 (0.69)	0.46 (1.18)	−0.21 (0.36)	2.96 (0.87)
rs1045599	3′	G/G	1.85 (1.98)	0.06 (0.70)	0.31 (1.18)	−0.15 (0.37)	3.00 (0.81)
(10)		A/G	1.95 (1.87)	0.19 (0.67)	0.63 (1.19)	−0.15 (0.36)	2.90 (0.94)
		A/A	1.92 (2.51)	0.23 (0.65)	0.74 (1.14)	−0.12 (0.39)	2.84 (0.93)
rs2286795	3′	G/G	1.84 (2.03)	0.04 (0.69)	0.25 (1.16)	−0.15 (0.38)	2.99 (0.83)
(11)		A/G	1.89 (1.69)	0.17 (0.66)	0.60 (1.19)	−0.13 (0.36)	2.93 (0.94)
		A/A	2.00 (2.51)	0.26 (0.67)	0.77 (1.14)	−0.15 (0.39)	2.82 (0.92)

Minor (variant) alleles are listed first. The rs7225585 (1) through the rs758857 (3) rows of the HOMA-IR column, the rs2779193 (2) through the rs8069362 (6) rows of the ln(IL-6) and ln(CRP) columns, and the rs2015353 (8) and rs2779211 (9) rows of the ln(CRP) 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 rs7225585 (1) through rs17715109 (7) rows of the ln(IL-2sR) 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 rises dramatically during inflammatory processes. We demonstrate six types of associations between diabetes/insulin resistance and A_{2B}Rs: *1*) diabetes is associated with elevated A_{2B}R mRNA expression in ECs and macrophages, *2*) diabetes is associated with elevated A_{2B}R-mediated cytokine production in ECs and macrophages, *3*) A_{2B}R activation in mice elevates fasted blood glucose levels, *4*) A_{2B}R activation in mice inhibits whole body glucose disposal, *5*) A_{2B}R blockade inhibits high-fat diet–induced blood glucose elevation, and *6*) A_{2B}R blockade inhibits diabetes-induced insulin-resistance during hyperinsulinemic-euglycemic glucose clamp. Our findings suggest that A_{2B}R 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 proinflammatiory 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 1 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) of five adjacent *ADORA2B* SNPs (numbered 2–6 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 A_{2B}R. 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

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

SNP	Site	*P* values			
		ln(IL-6)	ln(CRP)	ln(IL-2sR)	PAI-1
rs7225585					
Nondiabetics	5′	0.042‡ (0.043)*	0.215 (0.211)	0.629 (0.627)	0.333 (0.329)
Patients with diabetes		0.294 (0.288)†	0.526 (0.529)	0.055 (0.056)	0.315 (0.322)
rs2779193					
Nondiabetics	5′	0.782 (0.784)	0.844 (0.841)	0.177 (0.169)	0.785 (0.787)
Patients with diabetes		0.607 (0.607)	0.690 (0.688)	0.550 (0.550)	0.108 (0.108)
rs758857					
Nondiabetics	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					
Nondiabetics	intron 1	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)	0.015‡ (0.014)§
rs2041458					
Nondiabetics	intron 1	0.766 (0.761)	0.879 (0.876)	0.185 (0.194)	0.071 (0.074)
Patients with diabetes		0.241 (0.231)	0.839 (0.836)	0.051 (0.048)	0.044‡ (0.040)
rs8069362					
Nondiabetics	intron 1	0.755 (0.759)	0.328 (0.333)	0.317 (0.324)§	0.252 (0.251)
Patients with diabetes		0.079 (0.079)	0.813 (0.807)	0.009 (0.010)§	0.033‡ (0.034)
rs17715109					
Nondiabetics	intron 1	0.709 (0.714)	0.945 (0.943)	0.760 (0.755)	0.380 (0.377)
Patients with diabetes		0.835 (0.843)	0.230 (0.230)	0.779 (0.770)	0.452 (0.455)
rs2015353					
Nondiabetics	intron 1	0.463 (0.463)§	0.721 (0.716)§	0.081 (0.085)	0.443 (0.456)
Patients with diabetes		0.047‡ (0.047)§	0.001‡ (0.001)§	0.154 (0.148)	0.472 (0.466)
rs2779211					
Nondiabetics	intron 1	0.261 (0.253)	0.469 (0.456)§	0.216 (0.221)	0.731 (0.729)
Patients with diabetes		0.112 (0.113)	0.003‡ (0.002)§	0.825 (0.825)	0.534 (0.526)
rs1045599					
Nondiabetics	3′	0.499 (0.495)	0.372 (0.364)	0.584 (0.590)	0.763 (0.755)
Patients with diabetes		0.059 (0.058)	0.050‡ (0.049)	0.496 (0.490)	0.454 (0.454)
rs2286795					
Nondiabetics	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)	0.038‡ (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 $>10 \times$ 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}Rs 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}Rs. Deletion of the mouse A_{2B}R 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}R reduces inflammation. On the other hand, in this and several previous studies, activation of A_{2B}Rs increased IL-6 plasma levels in mice, and by several types of isolated cells (28), including macrophages (19) and dendritic cells (19,29). IL-$6^{-/-}$ mice develop mature onset obesity accompanied by abnormal glucose and fat metabolism (30). Although IL-6 is associated with diabetes, its actions are complex. IL-6 impairs insulin action in the liver and adipose tissue, but these effects depend on its concentration and duration of action (31). In skeletal muscle IL-6 has a dual role, acutely promoting insulin sensitivity but chronically resulting in insulin resistance through induction of JNK, suppressors of cytokine signaling-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 $A_{2B}R$ signaling can result in inhibition of lyphocyte 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 $A_{2B}R$ gene and selective $A_{2B}R$ blockade with ATL-801 implicate the $A_{2B}R$ 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 A_1R to increase glucose disposal after NECA administration to mice, possibly due to the known effect of A_1R blockade to increase pancreatic insulin secretion (35). Hyperinsulinemic-euglycemic glucose clamps in KKA^Y mice demonstrate that blockade of $A_{2B}R$ 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 $A_{2B}R$ 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 A_1, A_{2A}, and A_{2B} 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 $A_{2B}R$. 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 A_1Rs acutely counteracts insulin actions by stimulating catecholamine release and by counteracting the antilipolytic effect of A_1R 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^Y 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 A_{2B}Rs. 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 A_{2B}Rs, may contribute to the well-known effect of statins to provoke insulin resistance (45).

Diabetes and regulation of $A_{2B}R$ transcription. In the current study we demonstrate that diabetes triggers induction of $A_{2B}R$ mRNA in macrophages and ECs, resulting in increased cytokine production in response to $A_{2B}R$ activation. Analyses of the cloned human $A_{2B}R$ 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 $A_{2B}R$ 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 $A_{2B}R$ 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). $A_{2B}R$ signaling in nondiabetics due to low adenosine levels and low $A_{2B}R$ expression could render SNPs in *ADORA2B* that might influence $A_{2B}R$ signaling inconsequential in this population. In patients with diabetes, on the other hand, strong $A_{2B}R$ signaling may enhance functional consequences of *ADORA2B* SNPs. The findings of this study, in particular the induction of $A_{2B}R$ mRNA in ECs and macrophages from diabetic animals, are consistent with the possibility that one or more SNPs in *ADORA2B* influences $A_{2B}R$ mRNA expression. It will be of interest in future studies to determine whether *ADORA2B* genotypes are associated with $A_{2B}R$ mRNA expression in human monocytes.

In sum, the results of this study are consistent with the idea that diabetes enhances signaling through A_{2B}Rs both by elevating adenosine levels and by increasing the expression of the $A_{2B}R$. Our findings indicate that $A_{2B}R$ signaling can facilitate the release of proinflammatory cytokines from human macrophages and mouse ECs. Blockade or deletion of the $A_{2B}R$ 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* are 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 $A_{2B}R$ expression. It will be of interest to determine whether new potent and selective A_{2B} blockers that are currently in clinical development are effective in reducing obesity or insulin resistance in human disease.

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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 clamps. J.S.P. executed SNP analysis and interpretation. K.R. and B.F. provided transgenic mice and article editing. C.C.H. provided experimental design and wrote the article. S.S.R. executed SNP analysis and interpretation. J.K.K. executed and analyzed euglycemic clamps. J.L. provided experimental design and wrote the article.

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