Kir6.2 variant E23K increases ATP-sensitive potassium channel activity and is associated with impaired insulin release and enhanced insulin sensitivity in adults with normal glucose tolerance

Running Head: Kir 6.2 E23K variant and insulin secretion and action

Dennis T. Villareal1,* Joseph C. Koster,2* Heather Robertson,1 Alejandro Akrouh,1 Kazuaki Miyake,3 Graeme I. Bell,3 Bruce W. Patterson,1 Colin G. Nichols2 and Kenneth S. Polonsky1,2

*Both authors contributed equally to this research

1Department of Medicine and 2Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri and 3Department of Medicine, University of Chicago, Chicago, Illinois

Corresponding authors:
Kenneth S. Polonsky MD
Email: polonsky@wustl.edu
or

Colin G. Nichols PhD
Email: cnichols@wustl.edu

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**Background.** The E23K variant in the Kir6.2 subunit of the ATP-sensitive potassium (K\textsubscript{ATP}) channel is associated with increased risk of type-2 diabetes (T2D). The present study was undertaken to increase our understanding of the mechanisms responsible. To avoid confounding effects of hyperglycemia, insulin secretion and action were studied in subjects with the variant who had normal glucose tolerance.

**Research design and methods.** Nine subjects with the E23K genotype K/K and nine matched subjects with the E/E genotype underwent 5-h oral glucose tolerance test (OGTT), graded glucose infusion, and hyperinsulinemic-euglycemic clamp with stable-isotope-labeled tracer infusions to assess insulin secretion, action, and clearance. 461 volunteers consecutively genotyped for the E23K variant also underwent OGTT. Functional studies of the wild-type and E23K variant potassium channels were conducted.

**Results.** Insulin secretory responses to oral and intravenous glucose were reduced by ~40% in glucose-tolerant subjects homozygous for E23K. Normal glucose tolerance with reduced insulin secretion suggests a change in insulin sensitivity. The euglycemic-hyperinsulinemic clamp revealed that hepatic insulin sensitivity is ~40% greater in subjects with the E23K variant and these subjects demonstrate increased insulin sensitivity after oral glucose. The reconstituted E23K channels confirm reduced sensitivity to inhibitory ATP and increase in open probability, a direct molecular explanation for reduced insulin secretion.

**Conclusions.** The E23K variant leads to overactivity of the K\textsubscript{ATP} channel, resulting in reduced insulin secretion. Initially insulin sensitivity is enhanced thereby maintaining normal glucose tolerance. Presumably over time as insulin secretion falls further or insulin resistance develops, glucose levels rise resulting in T2D.
The ATP-sensitive potassium (K\textsubscript{ATP}) channel plays a central role in glucose-stimulated insulin secretion (GSIS). It is comprised of two subunits, an inward rectifier potassium channel (Kir6.2) and a sulfonylurea receptor (SUR1). An increase in the cytosolic ATP-to-ADP ratio in the pancreatic \( \beta \)-cell inhibits K\textsubscript{ATP} channel activity and stimulates insulin secretion, whereas a lowering of the ATP-to-ADP ratio restores channel activity and suppresses insulin release. Mutations in the genes encoding Kir6.2 (\textit{KCNJ11}) and SUR1 (\textit{ABCC8}) are associated with neonatal diabetes mellitus (NDM) and hyperinsulinism (1;2). A common amino acid polymorphism in Kir6.2, Glu23Lys (E23K) is associated with susceptibility to type 2 diabetes (T2D) (3). However, the mechanistic basis of this association and the phenotypic consequences \textit{in vivo} remain controversial. An early study observed no effects of the E23K variant on whole cell K\textsuperscript{+} currents (4). Conversely, more detailed studies report overactivity of E23K channels that is variably attributed to a decrease in channel sensitivity to inhibitory ATP (5) or an increase in ATP-sensitivity with an enhanced activation by FFAs (6). In both cases, net overactivity of the K\textsubscript{ATP} channel is predicted to suppress GSIS.

Although initial studies in humans showed no changes in insulin secretion, subsequent studies showed association of the E23K variant with reduced insulin secretion (7-9). If these studies are performed on diabetic subjects, reduced insulin secretion may result from the adverse effects of hyperglycemia on \( \beta \)-cell function. The present study was performed in order to provide quantitative indices of insulin secretion and define dose-response relationships between glucose and insulin secretion rate (ISR) at various glucose concentrations in subjects with the E23K genotype and normal glucose levels. The contribution of alterations in insulin clearance to insulin levels as a function of the E23K genotype was also examined.

It is unclear whether the K\textsubscript{ATP} regulates insulin sensitivity. Although an increase in insulin sensitivity is reported in animals with loss of K\textsubscript{ATP} channels (10), similar studies have not been performed with gain of channel activity. A study in human subjects suggested that the E23K genotype underlies an increase in insulin sensitivity (11) whereas insulin sensitivity was not altered (8;12-14) in other studies. The present study was undertaken to address these gaps in our understanding of the physiological mechanisms underlying susceptibility to T2D in subjects with the E23K variant. Since hyperglycemia can cause defects in insulin secretion, we focused on subjects with normal glucose tolerance. This allowed us to define the changes in insulin secretion and action that antedate the diabetes onset and are due to the effects of the E23K variant \textit{per se}. In order to resolve questions regarding the underlying mechanisms, we performed comprehensive assessments of the effect of E23K on K\textsubscript{ATP} activity.

**METHODS**

**Subjects.** Non-diabetic subjects <65 yr in good health and with stable weight for 6 months were recruited using advertisements. The studies were approved by the Human Research Protective Office.

**Study 1 (intensive metabolic studies).** Nine subjects with the K/K genotype and nine age-, sex-, and BMI-matched subjects with the E/E genotype participated in three separate protocols designed to test insulin secretion, action, and clearance. All subjects were unrelated and had no family history of T2D. Insulin secretion was assessed using the 5-h oral glucose tolerance test (OGTT) and graded-glucose infusion (GGI). Insulin sensitivity was assessed during the
hyperinsulinemic-euglycemic clamp and OGTT. Insulin clearance was assessed during OGTT and GGI.

**Study 2 (cross-sectional study).** 461 volunteers who responded to advertisements were genotyped for the E23K variant and underwent 5-h OGTT for assessments of insulin secretion, action, and clearance.

**Study protocols.** Subjects adhered to their regular diet and refrained from exercise for 3 days before the studies.

**5-h OGTT.** Participants ingested a 75-g glucose load. Blood samples were collected at -15, 0, 10, 20, 30, 60, 90, 120, 150, 180, 240, and 300 min after ingestion to determine plasma glucose, insulin, and C-peptide concentrations.

**GGI** involved the intravenous administration of glucose at increasing rates (1, 2, 3, 4, 6, and 8 mg/kg/min) for 40 min each (15;16). This protocol raises the plasma glucose concentration from basal to hyperglycemic levels and defines the dose-response relationships between glucose and insulin secretion.

**Hyperinsulinemic-euglycemic clamp with stable-isotope-labeled tracer infusion.** Studies were performed after an overnight fast as described (17;18). A primed (22.5 µmol/kg) constant (0.25 µmol·min⁻¹·kg⁻¹) infusion of [6,6⁻²H₂]glucose was started at 0700. After 3.5 h of tracer infusion, a hyperinsulinemic-euglycemic clamp was started and continued for 3.5 h. Insulin was infused at 40 mU·m⁻²·min⁻¹. Dextrose (20%) with [6,6⁻²H₂]glucose (~2.5%) was infused to maintain plasma glucose concentration at ~5.6 mmol/L. The infusion of [²H₂]glucose was decreased by 75% of basal during the clamp for the expected decline in hepatic glucose production.

**Body composition** was assessed using the same dual energy X-ray absorptiometry instrument in all subjects (Delphi 4500-W; Hologic Inc, Waltham, MA).

**Analyses.** The areas under the curve (AUC) were calculated using the trapezoid method (19). ISR was calculated using parameters for C-peptide kinetics and volume of distribution (20). Static, dynamic, and overall responsivity indices were calculated as reported (21). The GGI allowed the dose-response relationship between ISR and glucose over the physiological range to be determined. ISRs and glucose concentrations represented the average of the values between 10 and 40 min at each infusion rate. Mean ISR for each glucose infusion rate was plotted against the mean glucose concentration. The slope of the line relating these two variables provided a measure of the sensitivity of the β-cell to glucose. Peripheral insulin sensitivity was calculated from the hyperinsulinemic-euglycemic clamp (17;18;22). Basal endogenous glucose rate of appearance was calculated using Steele’s equations (20). Hepatic insulin sensitivity was assessed as the inverse of the product of the basal endogenous glucose production rate (µmol·kgFFM⁻¹·min⁻¹) and the fasting insulin concentration (pmol/l) x 1000 (23;24). Whole body insulin sensitivity was estimated using the oral glucose minimal model (25;26). Composite insulin sensitivity index was calculated using the method of Matsuda (23). Insulin clearance rate was calculated by dividing the 1) ISR AUC by the insulin AUC and the 2) C-peptide AUC by the insulin AUC (15;27).

**Biochemical measurements.** Plasma glucose was measured using a glucose analyzer (Yellow Springs Instruments, OH). Plasma insulin and C-peptide were measured using radioimmunoassays (Linco Research, St.Louis, MO).

**Genotyping.** DNA was prepared from peripheral blood lymphocytes and the KCNJ11 E23K polymorphism (dbSNP rs5219) typed using a TaqMan® SNP Genotyping Assay (Applied Biosystems, Foster City, CA).
Statistical Analyses. Group differences were compared using the t-test for unpaired samples for continuous variables and the Fisher’s exact test for categorical variables. Where appropriate we used analysis of variance and Tukey’s test for post-hoc analyses for continuous variables and chi-square test for categorical variables. Covariance analyses were used to adjust for age, race and BMI. SPSS version 15.0 (SPSS Inc, Chicago, IL) was used for all analyses. A $P$ value < 0.05 was considered to be statistically significant. Results are reported as mean±SEM.

In vitro studies of the E23K variant. Recombinant $K_{ATP}$ (Kir.6.2 (mouse) + SUR1 (hamster)) was transiently expressed in COSm6 cells as described (33). Of the missense variants reported in humans, the Kir.6.2 clone contained no variants, while the SUR1 clone contained the A1369S substitution.

Patch-clamp experiments were performed as described (33). Bath and pipette solutions (K-INT) contained (mM): 150 KCl, 10 HEPES, and 1 EGTA (pH 7.4). For experiments with MgATP, the free Mg$^{2+}$ concentration was kept at 2mM. The ATP dose-response was quantified by fitting the raw data with a Hill equation:

$$I_{rel} = \frac{1}{1 + \left( \frac{[ATP]}{K_{1/2,ATP}} \right)^H}$$

(Eq. 1)

where $I_{rel}$ is the current relative to that in the absence of ATP, [ATP] is the ATP concentration, $K_{1/2,ATP}$ is the half-maximal inhibitory ATP concentration, $H$ is the Hill coefficient which was allowed to vary. Dose-response curves for tolbutamide inhibition were described by the product of two Hill components (33), where $A$ represents the high affinity and $B$ the low affinity components:

$$I_{rel} = \left( \frac{1-L}{L} \right) \left( \frac{1}{1 + \left( \frac{[Tolb]}{K_{1/2,A}} \right)^H} + \left( \frac{[Tolb]}{K_{1/2,B}} \right)^H \right)$$

(Eq. 2)

where $[Tolb]$ is the tolbutamide concentration, $K_{1/2,A}$ and $K_{1/2,B}$ are the half-maximal inhibitory tolbutamide concentrations, $H_A$ and $H_B$ are the Hill coefficients, and $L$ is the fraction of current blocked with high affinity.

$86Rb^+$ efflux experiments were performed as described (33). Transfected cells were pre-incubated with or without metabolic inhibitors (2.5 µg/ml oligomycin plus 1 mM 2-deoxy-D-glucose). At selected time points, the solution was aspirated and replaced with fresh solution and the aspirated solution was counted in a scintillation counter. The rate constant of ATP-sensitive $K^+$-specific ($86Rb^+$) efflux ($k_2$) was obtained by fitting the data with a single-exponential equation:

$$\text{Relative flux} = 1 - \exp \left[ - (k_1 + k_2) * t \right]$$

(Eq. 3)

where the rate constant for nonspecific efflux ($k_1$) was obtained from untransfected cells.

Two approaches were used to estimate $P_{o,\text{zero}}$, the initial open probability (in zero ATP), of membrane patches containing multiple channels. (PIP2; Method I) PIP$_2$ was added to the patch until the current reached a saturating level (I$_{\text{PIP2}}$). This was assumed to represent a maximum $P_{o,\text{zero}}$ of ~0.97 (28). The fold increase in current was calculated (Fold increase = I$_{\text{PIP2}}$ / I$_{\text{initial}}$) and the $P_{o,\text{zero}}$ was estimated from the equation:

$$P_{o,\text{zero}} = 0.97 / \text{(Fold increase)}$$

(Eq. 4)

(NA; Method II) Mean $P_{o,\text{zero}}$ was estimated from stationary fluctuation analysis of macroscopic currents (29;30). Mean patch current (I), and variance ($\alpha^2$) in the absence of ATP were obtained by subtraction of the
mean current and variance in 5 mM ATP (i.e. all channels closed), respectively. Single channel current (i) was assumed to be -3.75 pA, corresponding to single channel conductance of 75 pS. \( P_{o,\text{zero}} \) was estimated from the following equation:

\[
\text{(Eq. 5)} \quad P_{o,\text{zero}} = 1 - (\alpha^2 / [i*I]).
\]

Channel density was calculated as \( I(P_{o,\text{max}} * i) \) from maximum patch current in zero ATP (I) and assuming a single channel current (i) = 3.75 pA at -50 mV.

RESULTS

Insulin secretion in subjects with E23K variant. We initially examined insulin secretion in subjects homozygous for the E23K variant, since they might exhibit the greatest difference if E23K genotype affected insulin secretion. The two groups were well-matched for age, sex, and BMI (Table 1). Both groups had normal glucose concentrations in the fasting state and following oral and intravenous glucose administration (Table 1, Figure 1). The respective AUCs for glucose between E/E and K/K subjects during the OGTT (17.6±0.1 vs. 18.7±0.1 x 10^4 min/mmol/l) and GGI (17.6±0.1 vs. 17.6±0.1 x 10^4 min/mmol/l) were not different (Figure 1). By contrast, the AUC for insulin in the OGTT (52.6±5.7 vs. 71.0±7.0 x 10^3 min/pmol/l) and GGI (24.5±2.1 vs. 36.1±4.8 x 10^3 min/pmol/l) was ~40% lower (\( P<0.05 \)) in the K/K group. The AUC for C-peptide was also reduced (\( P<0.05 \)) in the K/K subjects during both the OGTT (30.2±2.7 vs. 58.9±15.1 x 10^4 min/pmol/l) and GGI (26.4±2.1 vs. 36.9±3.3 x 10^4 min/pmol/l). Static, dynamic, and overall \( \beta \)-cell response to glucose were also ~40% lower (\( P<0.05 \)) in the K/K group based on the OGTT and GGI. In addition, the dose-response curve relating glucose and ISR during the GGI was shifted downward and to the right in the K/K group (Figure 2B).

Accordingly, the ISR AUC (17.2±1.2 vs. 26.1±0.5 x 10^3 pmol/l) and mean ISR (70.6±4.9 vs. 106.7±9.9 pmol/l/min) were lower (\( P<0.05 \)) in the K/K group. No significant differences were observed in insulin clearance rates in this study (\( P>0.05 \)).

Alterations in ATP-sensitivity of reconstituted E23K channels in vitro. To examine the molecular basis for the differences in insulin secretion, we transiently expressed Kir6.2 with residue 23 being either glutamate (E23) or lysine (K23) together with the SUR1 subunit and measured ATP-sensitivity in excised membrane patches (Figure 3A). Homomeric K23 channels (K/K) exhibit a modest, yet significant decrease in ATP inhibition compared to homomeric E23 (E/E) channels (K1/2,ATP =16 µM (n=28 patches) and 7.5 µM (n=24 patches), respectively. A similar relative shift in ATP sensitivities was observed in the presence of physiological (2 mM) Mg^2+ for E/E and K/K channels (K1/2,ATP =19 µM (n=19 patches) and 26 µM (n=17 patches), respectively). In contrast, the averaged \( K_{\text{ATP}} \) channel density was not different between cells expressing E/E or K/K channels (201±59 and 128±50 channels/patch, respectively; n=11-19 patches). To recapitulate the heterozygous E23K genotype (E/K), cells were transfected with a 1:1 mixture of E/E and K/K cDNAs. Since four subunits generate the channels, 5 different ratios of subunits will be present in the resultant channels (1/16 channels will be homozygous E/E and homozygous K/K). The ensemble of expressed channels display intermediate ATP-sensitivity (K1/2,ATP=10.0 µM (n=10 patches) compared to homomeric K/K and E/E channels (Figure 3B). Our data are similar to those reported by Schwanstecher and colleagues for recombinant K/K channels (5). For comparison, the dose-response curves are shown in Figure 3B for two Kir6.2 mutations that impair insulin release and underlie NDM. The I182V mutation underlies a transient
NDM, whereas the I296L mutation underlies a syndromic form of NDM (31;32). Importantly, the ATP-sensitivities of mutant channels correlate with the severity of the disease (K_{1/2,ATP}=39 \mu M for homomeric I182V (n=17 patches), and 771 \mu M for homomeric I296L channels (n=5 patches).

**Increased cellular activity of reconstituted E23K channels.** Kir6.2 channel activity in metabolically intact cells was screened by \(^{86}\text{Rb}\^-\text{-efflux from transfected COS}^m_6\text{ cells. Efflux from cells transfected with homomeric E/E channels was low under basal conditions, and was activated by metabolic inhibition to lower cellular [ATP]/[ADP]}(\text{Fig.4A}). \text{Rb}\^-\text{-efflux from cells transfected with recombinant K/K channels was also low under basal conditions and increased with metabolic inhibition. Quantitative estimation of K}_{\text{ATP}}\text{ conductance (see Methods) indicates that for K/K channels, basal conductance was a higher fraction of fully activated conductance than E/E channels (Fig. 4B). In the }\beta\text{-cell, the increased basal flux is expected to impair glucose-sensing and account for the association of K/K genotype with reduced insulin secretion.**

**E23K variant decreases ATP-sensitivity by stabilizing the open state of the channel.** Kir6.2 mutations can reduce ATP-sensitivity by directly reducing ATP-binding to the Kir6.2 subunit, or indirectly by affecting the intrinsic opening ability (28;33;34). In the latter case, an increase in the open probability (P_{o,zero}) decreases the frequency with which the channel enters the ATP-accessible closed state, resulting in a decrease in ATP-sensitivity. We have modeled this nonlinear relationship between P_{o,zero} and K_{1/2,ATP} (28) and this kinetic model describes the diabetes-causing effects of Kir6.2 mutations that underlie NDM (e.g. Q52R, I296L) (Figure 5C). To estimate the open probability of K}_{\text{ATP}}\text{ channels, both non-stationary noise analysis (NA) and phosphatidylinositol biphosphate (PIP\_2) application were used to independently examine K}_{\text{ATP}}\text{ channel gating in isolated membrane patches. As shown in Figure 5A, the estimated open probability (P_{o,zero}) of K/K channels in the absence of ATP (0.68±0.04 (NA method); 0.67±0.08 (PIP\_2 method)) is higher than that of E/E channels (0.49±0.05 (NA method); 0.46±0.06 (PIP\_2 method), P<0.05), and this increase can fully account for the shifted ATP-sensitivity (K_{1/2,ATP} = 9.4±1.6 \mu M (E/E), and 21.6±3.3 \mu M (K/K) from curve fit of individual membrane patches, P<0.01) (Figure 5B)). A similar increase in open probability was reported for K/K channels by Schwanstecher (5) using analysis of single channel records. That multiple methodologies reiterate the same findings strengthens the conclusion that the changes associated with the E23K variant are significant and real. As with more severe NDM, the E23K variant indirectly affects ATP-sensitivity by increasing the P_{o,zero} (Figure 5C). The predicted consequence will be reduced excitability of the }\beta\text{-cell, with increasingly severe consequences for insulin secretion (E23K<Q52R<I296L).**

**Sulfonylurea sensitivity is reduced in E23K channels.** Mutations in Kir6.2 which allosterically decrease ATP-sensitivity by stabilizing the open state of the channel also reduce high-affinity block by sulfonylureas, a feature of NDM-associated mutations (33). We next examined the effect of the first generation sulfonyurea, tolbutamide, on channel activity (Figure 3C). As shown in Figure 3D, homomeric E/E channels exhibit a typical biphasic block by tolbutamide with both a high-affinity (IC_{50}=1.1 \mu M) and a low-affinity site (IC_{50}=2 mM). The fractional block by the high-affinity, therapeutically relevant component is ~53%. For the K/K channels, the value for the high-affinity block (IC_{50}=1.0 \mu M) is unaltered; however, the fractional block is significantly decreased from 53% to 44%.
Insulin sensitivity in subjects with the E23K variant. The clinical findings described above of reduced insulin secretion with normal glucose concentrations suggest a simultaneous change in insulin sensitivity. Hyperinsulinemic-euglycemic clamp experiments revealed that hepatic insulin sensitivity was significantly greater (2.4±0.4 vs. 1.5±0.2 [1000/µmol/kgFFM/min•pmol/l]; P<0.05) in subjects with the K/K (Figure 6A) genotype. There was also a strong trend (0.19±0.03 vs. 0.14±0.2 µmol/min/kgFFM/pmol/l; P<0.10) for an increase in peripheral insulin sensitivity although the differences were not statistically significant. Similar trends were observed for whole body insulin sensitivity as assessed from the OGTT (Figure 6B). Glucose infusion rate, plasma glucose, insulin, glucagon, and tracer-tracee ratio are presented in supplementary material in the online appendix available at http://diabetes.diabetesjournals.org.

Confirmation of changes of insulin secretion and action. The human studies described above were conducted in small group of E/E or K/K subjects. To confirm the findings on insulin secretion and action in a larger cohort, we carried out 5-h OGTTs in 461 additional subjects (Table 2). Subjects were divided into three groups based on E23K genotype. The three groups had similar glucose concentrations fasting and 2-h post glucose challenge. The relative frequencies of E/K (40%) and K/K (13%) in our sample are comparable to those reported (7;8;35), conferring a relative diabetes risk of 1.15-1.65 (3). Consistent with the above findings, the K/K subjects had reduced insulin and C-peptide concentrations, reduced insulin secretory responses, reduced β-cell responsiveness to glucose, and enhanced insulin sensitivity and clearance rates. Interestingly, β-cell responsiveness was also lower in the E/K variant than the E/E.

DISCUSSION
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liver. This is the first study to report the coexistence of decreased insulin secretion with increased insulin action in subjects with the E23K polymorphism. Previous studies that evaluated insulin action yielded inconsistent results: one study suggested an increase in insulin sensitivity (11), while others found no effect (8;12;13). Moreover, findings from the hyperinsulinemic clamp suggested an increase in peripheral (skeletal muscle) insulin sensitivity, although this did not reach statistical significance ($P<0.10$), because of the small sample size. This interpretation is supported by the results obtained in the larger cohort study in which statistically significant increases in insulin sensitivity were observed after oral glucose ingestion. Although BMI was different between groups, measurements of total and regional fat (truncal fat) showed no differences. Nevertheless, because of the differences in BMI, we controlled for BMI using ANCOVA. The differences in insulin sensitivity remained significant ($P<.001$), despite similar and normal glucose tolerance.

The mechanistic basis of this change in insulin sensitivity is not clear. Kir6.2 is expressed in multiple tissues, including skeletal muscle, brain and heart. The increase in insulin sensitivity could be due to direct effects of the altered $K_{ATP}$ activity in one or more of these tissues, and a link between $K_{ATP}$ in the hypothalamus and the liver has recently been established (40). An increase in activation of $K_{ATP}$ channels in the hypothalamus decreases hepatic gluconeogenesis and this central effect could serve to counterbalance peripheral actions to maintain glucose homeostasis (40;41). It is interesting to note that basal and insulin-stimulated muscle glucose transport is increased in Kir6.2-null mice which suggest a role for this channel in the regulation of muscle glucose metabolism (10).

Whether the enhanced insulin sensitivity results from direct effects of $K_{ATP}$ channel activity in muscle, or is secondary to effects on insulin secretion is unclear. There is precedent for an increase in insulin sensitivity secondary to reduction in insulin secretion. In the evolution of type 1 diabetes, insulin secretion is reduced before glucose levels rise (42), and increased insulin sensitivity has been demonstrated in normoglycemic carriers of HNF1α mutations with reduced insulin secretion prior to diabetes onset (43). These observations, coupled with those of the present study, suggest that in the evolution of T2D, increased insulin sensitivity may compensate for a reduction in insulin secretion resulting in normal glucose tolerance. Over time glucose intolerance develops due to progression in the severity of the secretion defect and/or exposure to factors that reduce insulin sensitivity. However, we also cannot completely exclude the likelihood that these E23K variants have not developed diabetes because of their increased insulin sensitivity. In such case, it might reflect a feature of non-diabetic E23K variants rather than of subjects with E23K variants per se. Although Kir6.2 levels are high in pancreatic α-cells, we did not find differences in glucagon secretion. An additional new finding is the association of E23K variant with an increase in insulin clearance, as demonstrated in study 2. An increase in insulin clearance may contribute to the reduction in peripheral insulin concentrations that is due largely to the decrease in insulin secretion. The liver is the major site of insulin clearance under physiologic circumstances and this change in insulin clearance is likely due to a change in hepatic insulin metabolism (44). A link between hepatic insulin receptor-binding and action and degradation by the liver has been reported (45).

**Molecular basis of the E23K phenotype.** In heterologous expression studies, reconstituted E23K channels exhibit a mild yet significant decrease in ATP-
sensitivity, and a relative increase in basal activity in the intact cell. In the β-cell, decreased ATP inhibition and consequent channel overactivity, is predicted to suppress glucose-sensing. In contrast to activating mutations in Kir6.2 that underlie NDM (33), the E23K variant has a less radical effect on channel activity and is associated with T2D.

The molecular consequence of the E23K variant has been controversial. Given the high K\textsubscript{ATP} density in β-cells, it is predicted that a change in K\textsubscript{ATP} channel activity (<1%) could significantly affect insulin secretion and, therefore, subtle effects of E23K on channel activity could be physiologically relevant. In support of the conclusion that E23K does not alter channel activity, whole cell K’-currents were similar in Xenopus oocytes expressing E/E or K/K channels (4). A subsequent study reported an increase in ATP-sensitivity of K/K channels, relative to E/E, but a subsequent decrease in ATP-inhibition upon application of long-chain acyl-CoA (6). The most detailed study was carried out by Schwanstecher and colleagues (5;46). Our data parallel their initial findings of ~2-fold reduction in ATP-sensitivity and increase in open probability, and reduced SU sensitivity (46), associated with the E23K variant. The similarity of our findings, in a completely independent study, utilizing different methodologies to assess open probability, strongly supports the conclusion that the E23K variant decreases ATP-inhibition of the K\textsubscript{ATP} channel.

Mechanistically, the observed increase in open probability can account for both the reduced insulin secretion and decrease in sulfonylurea-inhibition of E23K channels. Whether the E23K variant affects sulfonylurea-dosing is unknown, but it is notable that the E23K variant is associated with risk for secondary failure to sulfonylureas in T2D patients (47;48) and human islets isolated from E23K donors (E/K and K/K) exhibit a decrease in sulfonylurea-induced insulin secretion (47).

**CONCLUSIONS**

Subjects with the Kir6.2 E23K variant have multiple insulin secretory defects to glucose and decreased responsiveness of the β-cell over a physiologic range of glucose concentrations and these can be explained by the molecular properties of the E23K channels. These defects in insulin secretion are accompanied by an increase in insulin sensitivity, possibly a compensatory response to reduced insulin secretion. The β-cell secretory defects are present prior to diabetes onset and are likely responsible for the increased risk of T2D in subjects with the at-risk E23K genotypes, if superimposed on lifestyle factors causing insulin resistance.

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Table 1. Characteristics of Study Participants (Study 1)

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<tr>
<td>Fat free mass (kg)</td>
<td>43.7 ± 3.6</td>
<td>49.2 ± 3.7</td>
<td>0.30</td>
</tr>
<tr>
<td>Truncal fat (kg)</td>
<td>13.4 ± 1.8</td>
<td>10.0 ± 1.7</td>
<td>0.20</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>5.1 ± 0.2</td>
<td>5.2 ± 0.1</td>
<td>0.73</td>
</tr>
<tr>
<td>2-Hour Glucose (mmol/l)</td>
<td>6.9 ± 0.3</td>
<td>6.8 ± 0.3</td>
<td>0.62</td>
</tr>
<tr>
<td>Hemoglobin A1C (%)</td>
<td>5.6 ± 0.1</td>
<td>5.5 ± 0.2</td>
<td>0.72</td>
</tr>
</tbody>
</table>

Values are mean ± SEM
Table 2. Characteristics of Study Participants (Study 2)

<table>
<thead>
<tr>
<th></th>
<th>E/E</th>
<th>E/K</th>
<th>K/K</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>216</td>
<td>184</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>Women (%)</td>
<td>158 (73)</td>
<td>120 (65)</td>
<td>45 (74)</td>
<td>0.40</td>
</tr>
<tr>
<td>Caucasian (%)</td>
<td>127 (59)</td>
<td>155 (84)</td>
<td>55 (90)</td>
<td>0.001</td>
</tr>
<tr>
<td>Age (years)</td>
<td>38.0 ± 0.9</td>
<td>38.2 ± 1.1</td>
<td>36.6 ± 12.4</td>
<td>0.74</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>29.5 ± 0.5</td>
<td>27.5 ± 0.5*</td>
<td>27.1 ± 0.9*</td>
<td>0.001</td>
</tr>
<tr>
<td>Fat mass (kg)</td>
<td>33.5 ± 0.7</td>
<td>31.9 ± 0.7</td>
<td>31.8 ± 1.3</td>
<td>0.23</td>
</tr>
<tr>
<td>Truncal fat (kg)</td>
<td>13.2 ± 0.5</td>
<td>12.2 ± 0.4</td>
<td>11.4 ± 0.9</td>
<td>0.16</td>
</tr>
<tr>
<td>Hemoglobin A1C (%)</td>
<td>5.6 ± 0.0</td>
<td>5.5 ± 0.0*</td>
<td>5.5 ± 0.1*</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Oral glucose tolerance test

<table>
<thead>
<tr>
<th></th>
<th>E/E</th>
<th>E/K</th>
<th>K/K</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>5.3 ± 0.1</td>
<td>5.1 ± 0.0</td>
<td>5.2 ± 0.1</td>
<td>0.28</td>
</tr>
<tr>
<td>2-H glucose (mmol/l)</td>
<td>7.8 ± 0.1</td>
<td>7.6 ± 0.1</td>
<td>7.4 ± 0.2</td>
<td>0.43</td>
</tr>
<tr>
<td>Glucose AUC x 10⁴ (min/mmol/l)</td>
<td>19.6 ± 0.2</td>
<td>19.1 ± 0.3</td>
<td>18.8 ± 0.4</td>
<td>0.34</td>
</tr>
<tr>
<td>Insulin AUC x 10³ (min/pmol/l)</td>
<td>90.2 ± 4.2</td>
<td>73.6 ± 3.5</td>
<td>62.5 ± 4.9*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C-peptide AUC x 10⁴ (min/pmol/l)</td>
<td>51.6 ± 1.4</td>
<td>48.9 ± 1.3</td>
<td>43.4 ± 2.2*</td>
<td>0.010</td>
</tr>
</tbody>
</table>

Insulin sensitivity (SI)

<table>
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<th>E/K</th>
<th>K/K</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SI_MM x 10⁻⁴ (dl/kg/min per pmol/l)†</td>
<td>1.7 ± 0.1</td>
<td>1.8 ± 0.7</td>
<td>2.6 ± 0.2†</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SIComposite‡</td>
<td>5.1 ± 0.2</td>
<td>5.8 ± 0.3</td>
<td>6.7 ± 0.5*</td>
<td>0.007</td>
</tr>
</tbody>
</table>

Insulin secretion

<table>
<thead>
<tr>
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<th>E/K</th>
<th>K/K</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin secretory rate, ISR AUC x 10³</td>
<td>30.0 ± 0.9</td>
<td>28.1 ± 0.8</td>
<td>25.4 ± 1.2*</td>
<td>0.01</td>
</tr>
<tr>
<td>β-cell responsivity, Φ₀ (10⁹ min⁻¹)</td>
<td>13.7 ± 0.84</td>
<td>13.3 ± 0.4*</td>
<td>11.5 ± 0.7*</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Insulin clearance (IC)

<table>
<thead>
<tr>
<th></th>
<th>E/E</th>
<th>E/K</th>
<th>K/K</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC₁ (ISR AUC/Insulin AUC)</td>
<td>2.8 ± 0.1</td>
<td>3.1 ± 0.1*</td>
<td>3.2 ± 0.1*</td>
<td>0.001</td>
</tr>
<tr>
<td>IC₂ (C-peptide AUC/Insulin AUC)</td>
<td>47.8 ± 1.1</td>
<td>53.3 ± 1.4*</td>
<td>55.9 ± 2.3*</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. Covariance analyses was used to adjust for age, race, and BMI
*P <.05 vs. E/E, †P <.05 vs. E/E and E/K using Tukey’s test for posthoc analyses
†SI_MM = insulin sensitivity from glucose minimal model (25)
‡SIComposite = insulin sensitivity from Matsuda (23)
Figure Legends

Figure 1. Plasma glucose, insulin, and C-peptide concentrations during the (A) oral glucose tolerance test and (B) intravenous graded glucose infusion in the K/K (open circles) and E/E groups (closed circles). *P values indicate the significance of the differences between area under the curve values between groups. Values are mean ±SEM.

Figure 2. (A) Insulin secretory indices during the oral glucose tolerance test (OGTT) and intravenous graded glucose infusion (GGI) in the K/K (open bars) and E/E groups (closed bars). *P < 0.05 for the differences between groups. (B) Insulin secretory rate-glucose dose-response curve as constructed from the intravenous graded glucose infusion in the K/K (open circles) and E/E groups (closed circles). P value indicates the significance of the differences between area under the curve values between groups. Values are mean ± SEM.

Figure 3. Reduced ATP- and sulfonyurea-sensitivity of mutant E23K channels. (A) Representative currents (at –50 mV) from inside-out membrane patches from COS cells expressing K<sub>ATP</sub> (Kir6.2 + SUR1): homomeric E23 channels (E/E), K23 channels (K/K), or heteromeric E23 and K23 channels (E/K). Patches were exposed to differing [ATP] and baseline current was determined by exposure to ATP (5mM). (B) Steady-state dependence of membrane current on [ATP] (relative to current in zero ATP (Irel)) for E23 and K23-containing channels. K<sub>1/2,ATP</sub> = 7.5 µM (E/E), and 16 µM (K/K). Data points represent mean ± SEM. (n = 24-28 patches). The fitted lines correspond to least squares fits of a Hill equation (see Methods). **P < 0.01 as compared to E/E channels by unpaired student’s t-test (two-tailed, assuming equal variance). (C) Representative currents recorded from inside-out membrane patches containing homomeric E/E or mutant K/K channels at -50 mV and in response to varying [tolbutamide]. Zero channel current was determined by application of ATP (5 mM). (D) Steady-state dependence of current on [tolbutamide] (relative to current in zero tolbutamide (Irel)) for E/E and K/K variant channels (from records such as those shown in C). Data points represent the mean ± SEM (n= 6-19 patches). For all channels, the lines are fits of the product of two Hill components, each of the form (Irel = 1/(1+(Tolb/K<sub>1/2</sub>))<sup>H</sup>) with H fixed at 1.3 in each case (see Methods). The relative fraction and K<sub>1/2</sub> values of each component were varied. The high-affinity component was 53 and 44% for wild-type, and K/K, channels, respectively. *P < 0.05 as compared to wild-type K<sub>ATP</sub> by unpaired t test. The shaded box shows the reported range of serum tolbutamide concentrations from a cohort of 37 type 2 diabetics receiving sulfonylurea therapy (49).

Figure 4. Increased basal activity in intact cells expressing K/K variant channels. (A) Representative efflux of <sup>86</sup>Rb<sup>+</sup> as a function of time in basal conditions or in the presence of metabolic inhibition for reconstituted E/E and variant K/K channels, and untransfected controls. (C) Ratio of K<sub>ATP</sub>-dependent efflux rate constant (k2) in basal conditions relative to metabolic inhibition (MI) for E/E, or homomeric
Kir 6.2 E23K variant and insulin secretion and action

K/K channels (see Methods). Graphs show compiled data (mean ± s.e.m.) from 6 experiments in which each transfection was done in triplicates. *P < 0.05 as compared to E/E channels by paired, one-tailed Student’s t test.

**Figure 5** An increase in maximum open probability underlies the reduced ATP-sensitivity of K/K variant channels. (A) Open probability in zero ATP (P₀,zero) calculated using PIP₂ method and noise analysis (NA)(see Methods) for membrane patches expressing either homomeric E/E or K/K channels. Data points represent mean ± SEM. (n = 21-44 patches for (NA) method; n=6-8 patches for PIP2 method) *P < 0.05 and **P < 0.01 by two-tailed student’s t test assuming equal variance. (B) Relationship between P₀,zero (calculated using the NA method) and ATP-sensitivity (K₁/₂,ATP) for individual membrane patches expressing E/E or K/K channels, together with averaged data (triangles). For E/E: K₁/₂,ATP = 9.4 ± 1.6 µM, P₀,zero =0.49 ± 0.05 (n=18 patches). For K/K: K₁/₂,ATP = 21.6 ± 3.3 µM, P₀,zero =0.68 ± 0.04 (n=18 patches). **P < 0.01 for both K₁/₂,ATP and P₀,zero values of E/E compared to K/K channels(unpaired t-test). Data points represent mean ± SEM. (C) Relationship between P₀,zero and K₁/₂, ATP [mM]. Solid line represents prediction of kinetic model II (inset) of Enkvetchakul et al. (2000) (28) Symbols represent data points as in B, together with mean values for Q52R, and I296L channels The key feature of the model is that ATP acts by binding to a closed state and, in consequence, ATP-sensitivity is reduced by shifting the equilibrium between the Cin and O states toward the open state (increasing KCO)

**Figure 6.** Hepatic insulin sensitivity and peripheral insulin sensitivity from the hyperinsulinenimic-euglycemic clamp with stable-isotope-tracer infusion (A) and whole body insulin sensitivity from the OGTT (B) in the K/K (open bars) and E/E groups (closed bars). Values are mean ± SEM. *P < 0.05 for the differences between groups.
Figure 1
Figure 2

A

- Static β-cell responsivity
  - OGTT vs. GGI

- Dynamic β-cell responsivity
  - OGTT vs. GGI

- Overall β-cell responsivity
  - OGTT vs. GGI

B

- Insulin Secretory Rate (pmol/L·min)
  - Plasma Glucose (mmol/L)

P < 0.05
Figure 5
Figure 6

A

Hepatic Insulin Sensitivity

Peripheral Insulin Sensitivity

K/K  E/E  K/K  E/E

B

Whole Body Insulin Sensitivity

K/K  E/E

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