

Brief Genetics Report

In Vitro and In Vivo Studies of a Naturally Occurring Variant of the Human p85 α Regulatory Subunit of the Phosphoinositide 3-Kinase

Inhibition of Protein Kinase B and Relationships With Type 2 Diabetes, Insulin Secretion, Glucose Disappearance Constant, and Insulin Sensitivity

Lars Hansen¹, Björn Zethelius², Lars Berglund², Richard Reneland², Torben Hansen¹, Christian Berne³, Hans Lithell², Brian A. Hemmings⁴, and Oluf Pedersen¹

In humans, the Met326Ile missense variant of the p85 α regulatory subunit of the phosphoinositide 3-kinase (PI3K) has been associated with either significant reductions in glucose effectiveness and intravenous glucose tolerance in Caucasians or a significantly higher insulin secretory response in Pima Indians. In the present study, we genotyped 1,190 Caucasian males to evaluate the impact in vivo of the Met326Ile variant of the p85 α subunit of PI3K on the acute insulin response, intravenous glucose tolerance, insulin-mediated glucose uptake, and the prevalence of type 2 diabetes after 20 years of follow-up. We also expressed the variant in vitro to evaluate the impact on insulin-stimulated activation of protein kinase B (PKB). The Met326Ile variant of p85 α was not associated with type 2 diabetes or with alterations in insulin secretion, insulin sensitivity, or intravenous glucose tolerance in vivo. Expressed in vitro, the Ile326 and the Met326 variant acted equally as a dominant-negative and prevented (60–70% inhibition) insulin-mediated activation of PKB by inhibiting the phosphorylation of PKB at Thr308. We conclude that the Met326Ile variant of the p85 α regulatory subunit of PI3K is likely to be as functionally normal in vivo as in vitro. *Diabetes* 50:690–693, 2001

From the ¹Steno Diabetes Center, Copenhagen, Denmark; the ²Department of Public Health and Caring Sciences/Section of Geriatrics and the ³Department of Medical Sciences, University of Uppsala, Sweden; and the ⁴Friedrich Miescher-Institut, Basel, Switzerland.

Address correspondence and reprint requests to Lars Hansen, MD, Steno Diabetes Center, Niels Steensens Vej 2, DK-2820 Gentofte, Copenhagen, Denmark. E-mail: larh@hagedorn.dk.

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CMV, cytomegalovirus; HA, hemagglutinin; IMGU, insulin-mediated glucose uptake; IVGTT, intravenous glucose tolerance test; K_g , glucose disappearance constant; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; S_g , glucose effectiveness constant; ULSAM, Uppsala Longitudinal Study of Adult Men.

In the search for biological evidence to explain the apparent heritability of the phenotype for insulin resistance in general and late-onset type 2 diabetes in particular (1), genes encoding proteins involved in the insulin-signaling cascade (2) have been regarded as potential candidate genes by many investigators (3,4). The results of subsequent mutational analysis of candidate genes in cohorts of insulin-resistant type 2 diabetic patients have been negative in the majority of studies (4). In a few studies, one or more variants of candidate genes have been identified, suggesting association to either the disease (5,6) or an insulin-resistant phenotype (7,8) within the same population. Because the phosphoinositide 3-kinase (PI3K) plays a pivotal role in both the metabolic (glucose uptake and glycogen synthesis) and the mitogenic actions induced by insulin at the cellular level, it is a logical candidate for a diabetogene (2,9). Although things have been complicated by the existence of different isoforms, splice variants, and regulatory and catalytic subunits of the PI3K, the p85 α regulatory subunit has been analyzed for mutations in two different ethnic populations of late-onset type 2 diabetic patients (10,11). The mutation screening of p85 α in Caucasian patients with type 2 diabetes (10) and in Pima Indians (11) identified the same Met326Ile variant with an allelic frequency of 16% in Caucasians and 25% in Pima Indians. In young healthy Caucasians, the homozygous Ile326Ile genotype was associated with a 40% decrease in intravenous glucose disappearance (K_g) and a 23% reduction in glucose effectiveness (S_g), as estimated from a tolbutamide-modified intravenous glucose tolerance test (IVGTT), according to Bergman's minimal model, when compared with the wild-type Met326Met or heterozygous Met326Ile genotypes (10). In female Pima Indians, the homozygous Ile326Ile variant was associated with a lower prevalence of type 2 diabetes and an increased first-phase insulin secretory response

TABLE 1

K_g and logarithm of acute insulin response at age 50 years and IMGU and BMI at age 70 years in Swedish men, according to genotype of the Met326Ile variant of the p85 α regulatory subunit of PI3K

Genotype	Met326Met (<i>n</i> = 875)	Met326Ile (<i>n</i> = 291)	Ile326Ile (<i>n</i> = 24)	<i>P</i>
BMI at 70 years (kg/m ²)	26.3 ± 3.4 (<i>n</i> = 868)	26.3 ± 3.4 (<i>n</i> = 289)	25.8 ± 3.5 (<i>n</i> = 24)	0.8
K_g (mmol · l ⁻¹ · min ⁻¹)‡	<u>Met326Met + Met326Ile*</u> 1.8 ± 0.7 (<i>n</i> = 917)	<u>Met326Ile + Ile326Ile†</u> 1.8 ± 0.7 (<i>n</i> = 237)	1.8 ± 0.7 (<i>n</i> = 17)	0.8*
Log (acute insulin response 0–8 min)‡	5.0 ± 0.7 (<i>n</i> = 942)	5.0 ± 0.7 (<i>n</i> = 247)	5.0 ± 0.6 (<i>n</i> = 19)	0.8* 1.0†
IMGU (mg · kg ⁻¹ · min ⁻¹ · [mU/l] ⁻¹ · 100)§	5.0 ± 2.5 (<i>n</i> = 1113)	5.1 ± 2.5 (<i>n</i> = 299)	5.2 ± 2.2 (<i>n</i> = 24)	0.7* 0.7†

Data are means ± SD. *Heterozygous and wild-type carriers are analyzed together against homozygous carriers in a recessive genetic model as previously described (10,11); †homozygous and heterozygous carriers are analyzed together against wild-type carriers in a dominant/codominant model; ‡measured during an IVGTT (0.5 g glucose/kg body wt); *P* values adjusted for BMI and fasting serum insulin: *P* = 0.9 (recessive model) and *P* = 1.0 (dominant model); §measured during the last 60–120 min of steady state of a 120-min hyperinsulinemic-euglycemic clamp (56 mU insulin · min⁻¹ · m⁻²).

during an IVGTT when compared with the wild-type Met326Met or heterozygous Met326Ile genotypes (11), whereas in Japanese subjects there were no associations with type 2 diabetes or indications of changes in insulin sensitivity (12).

The purposes of the present study were 1) to evaluate the impact of the Met326Ile variant of the p85 α subunit of PI3K on acute insulin response, intravenous glucose tolerance, and insulin-mediated glucose uptake (IMGU) during a hyperinsulinemic-euglycemic clamp; 2) to determine whether this variant was associated with impaired glucose tolerance or overt type 2 diabetes after 20 years of follow-up; and 3) to evaluate in vitro the biological function of the Met326Ile variant on insulin action downstream of PI3K in the insulin-signaling cassette.

For the human studies, the study group comprised 1,190 men participating in the Uppsala Longitudinal Study of Adult Men (ULSAM), a population-based study of diabetes and cardiovascular disease in men that has been described previously (13). The allelic frequency of the Ile326 variant was 14% (95% CI 13–15%), which is in accordance with earlier findings in Caucasians (10), and the distribution of genotypes was in Hardy-Weinberg equilibrium (data not shown). Data analysis showed that neither insulin sensitivity, as estimated by IMGU, during the clamp at 70 years of age nor the K_g or the acute insulin response (0–8 min) during the IVGTT at 50 years of age differed significantly between homozygous carriers of the Ile326Ile variant and the Met326Met/Met326Ile carriers (Table 1). Furthermore, the Ile326Ile variant was not associated with type 2 diabetes, and after 20 years of follow-up, the prevalence of type 2 diabetes was 4% among homozygous (Ile326Ile) carriers vs. 16% in the wild-type/heterozygous (Met326Met/Met326Ile) carriers (*P* = 0.2) and 17 vs. 28% in the combined group of diabetes and impaired glucose tolerance (RR = 1.69, 95% CI 0.69–4.17, *P* = 0.4) (Table 2).

To further evaluate the biological function of the Met326Ile variant of the p85 α regulatory subunit of PI3K, either Met326 or Ile326 clones were transiently coexpressed together with hemagglutinin (HA)-tagged PKB α in HEK293 cells. Coexpression of either form of p85 α equally inhibited insulin-stimulated activation of HA-PKB α , with a maximal 60–70% inhibition within 5 min of insulin stimu-

lation (Fig. 1A and B). Both forms of p85 α expressed equally, and coexpression of p85 α did not affect expression levels of HA-PKB α as estimated by Western analysis and blotting with 12CA5 anti-HA or anti-p85 α antibodies (Fig. 2, panels A and B). Blotting with PKB anti-phospho-Thr308-specific antibodies showed that the inhibition of PKB α was paralleled by less effective phosphorylation of this residue (Fig. 2, panel C).

We have used the ULSAM data in a cross-sectional design to investigate the impact of the Ile326Ile variant of the p85 α regulatory subunit of PI3K on the K_g and the acute insulin response during an IVGTT and on the IMGU during a hyperinsulinemic-euglycemic clamp. In contrast to the previous studies (10,11), this variant was not associated with any changes in acute insulin response (10) or K_g (11). These discrepancies might be explained by fewer homozygous subjects in the Pima Indian (*n* = 5) (11) and the Danish studies (*n* = 8) (10) compared with 17 and 19 subjects in the present study (Table 1). In addition, the K_g was calculated between the two time points at 8 and 19 min during the IVGTT in the study of 380 young healthy Danes (10), whereas the time points 6, 20, 30, 40, 50, and 60 min used in the present study may give a more accurate estimation of K_g . Finally, the Ile326Ile variant was not associated with type 2 diabetes in these 1,190

TABLE 2

Prevalence of type 2 diabetes and impaired glucose tolerance during a 20-year follow-up period (from age 50 to 70 years) in 1,157 Swedish men who underwent an oral glucose tolerance test, according to genotype of the Met326Ile variant of the p85 α regulatory subunit of PI3K

Genotype	Phenotype			Total
	Type 2 diabetes*	Impaired glucose tolerance†	Normal glucose tolerance	
Ile326Ile	1 (4)	3 (13)	20 (83)	24 (100)
Met326Ile‡	35 (13)	36 (13)	208 (74)	279 (100)
Met326Met‡	137 (16)	107 (13)	610 (71)	854 (100)

Data are *n* (%). **P* = 0.2 (Fischer's exact test); †*P* = 0.4 (Fischer's exact test) for type 2 diabetes or impaired glucose tolerance combined; ‡heterozygous and wild-type carriers are analyzed together, as previously described (10,11).

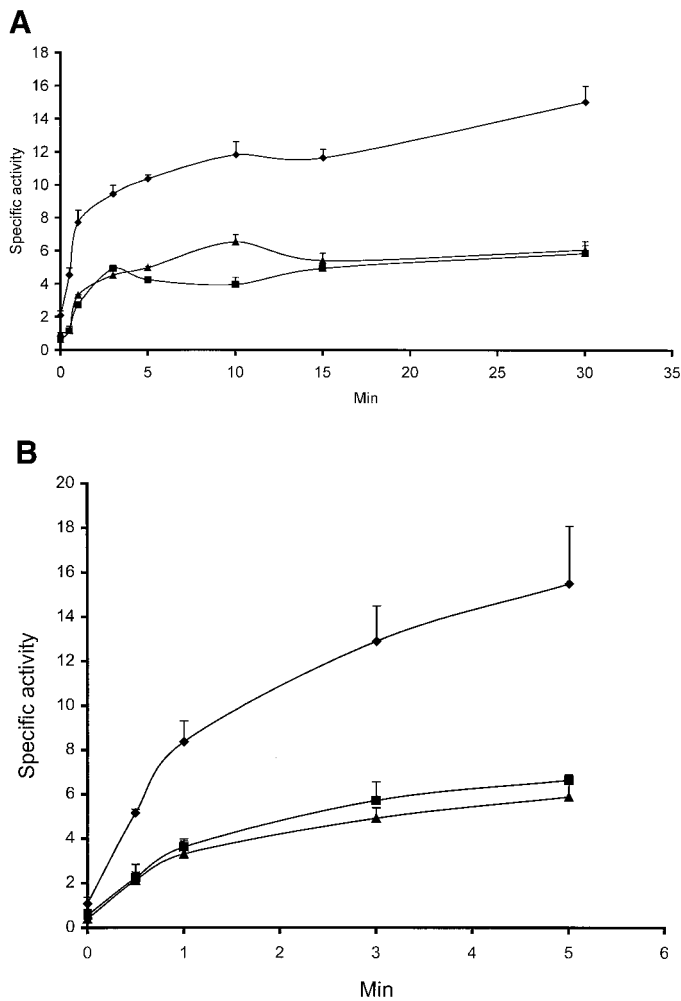


FIG. 1. A: Time-course from 0 to 30 min of insulin-stimulated (500 nmol/l) HA-PKB activity expressed in HEK293 cells alone (♦) or together with either Met326 (■) or Ile326 (▲) variants of the p85 α subunit of PI3K. In vitro activities (pmol incorporated [γ-³²P]ATP/mg protein used for immunoprecipitation/min) of immunoprecipitated HA-PKB α were measured as described in RESEARCH DESIGN AND METHODS. **B:** Time-course from 0 to 5 min of insulin-stimulated PKB activity expressed in HEK293 cells alone (♦) or together with either Met326 (■) or Ile326 (▲) variants of the p85 α subunit of PI3K. In vitro activities of immunoprecipitated HA-PKB α (pmol incorporated [γ-³²P]ATP/mg protein used for immunoprecipitation/min) were measured as described in RESEARCH DESIGN AND METHODS.

Swedish males after 20 years of follow-up from the age of 50 years to the age of 70 years.

In vitro the Met326 and Ile326 variants of p85 α have been shown to have similar basal binding and recruitment to phosphotyrosine-containing immunoprecipitates after insulin stimulation when coexpressed with the catalytic subunit p110 α in HEK293 cells (14); the catalytic activities of the Met326- or Ile326-containing phosphotyrosine complexes were identical (14).

Our findings of an equal inhibition of the downstream activation of PKB by the Met326 and Ile326 variants of the p85 α also suggest equal binding and recruitment of these variants to tyrosine-phosphorylated proteins after insulin stimulation. The dominant-negative effect of overexpressing full-length p85 α on activation of PKB is in the same order of magnitude as when overexpressing the dominant-negative form (Δ p85) of p85 α (15,16). Interestingly, the same inhibition of PKB activity was reported when the

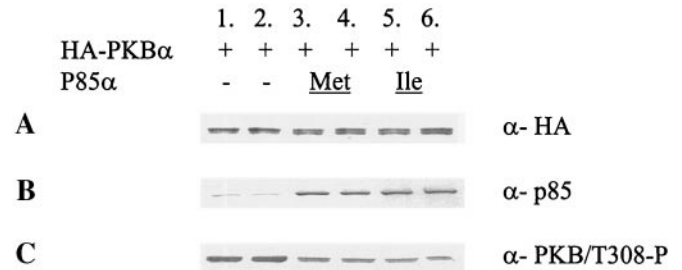


FIG. 2. Western blotting of total cell lysates from HEK293 expressing HA-PKB α alone (lanes 1 and 2), HA-PKB α and the Met326 variant of p85 α subunit of PI3K (lanes 3 and 4), or HA-PKB α and the Ile326 variant of p85 α subunit of PI3K (lanes 5 and 6). **Panel A:** Blotting with anti-HA antibody. **Panel B:** Blotting with anti-p85 α antibody. **Panel C:** Blotting with anti-phospho-Thr308/PKB antibody (as described in RESEARCH DESIGN AND METHODS).

COOH-terminal SH2 domain of the p85 was coexpressed (17), and this inhibition could not be rescued by coexpressing the constitutively active PI3K p110-CAAX (17); thus, the dominant-negative effect might be independent of the lipid kinase activity of the PI3K holoenzyme. Because all of these different p85 α constructs contain the same COOH-terminal SH2 domain, it suggests that the dominant-negative effect of coexpressing either of the p85 α variants is caused by occupying binding sites at the level of the plasma membrane, thus preventing PKB to translocate to the membrane and become phosphorylated on Thr308. Normally, maximal PKB accumulation at the plasma membrane occurs within 5 min of insulin/IGF-1 stimulation (15), the time window also chosen in the present studies. We and others have shown that transient overexpression of p85 α inhibits the effects of endogenous PI3K activity. However, we cannot exclude the possibility that if we had selected for stably transfected clones, some opportunistic compensation might have resulted in complete rescue or even enhanced PKB activity. Knockout mice missing the full-length p85 α subunit of the PI3K show compensation by cellular recruitment of the p50 α splice form, resulting in increased insulin sensitivity and hypoglycemia (18).

Based on the present studies in humans as well as in appropriate expression systems, we conclude that the Ile326 variant of the p85 α regulatory subunit of the PI3K is most likely to represent a functionally normal variant.

RESEARCH DESIGN AND METHODS

Subjects. The present study included 1,190 men participating in the Uppsala Longitudinal Study of Adult Men (ULSAM), a population-based study of diabetes and cardiovascular disease in men, described previously (13). Briefly, all men born between 1920 and 1924 who were residents of the municipality of Uppsala were invited to participate in a health survey between 1970 and 1973 (baseline: 50 years of age). Altogether, 2,322 of 2,841 men attended the survey; of this initial cohort of 2,322 men, 615 were born in the Uppsala Academic Hospital, 1,585 were born elsewhere in Sweden, and 122 were born outside of Sweden. An IVGTT was performed in 1,792 50-year-old men at baseline. The serum insulin concentrations during the IVGTT were measured in duplicate of blood samples drawn before and 4, 6, 8, and 60 min after the start of the glucose injection (0.5 g/kg body wt), and the acute insulin response was expressed as the sum of the serum insulin concentrations determined at 4, 6, and 8 min. For the estimation of the K_{it} , blood glucose was measured before and at 6, 20, 30, 40, 50, and 60 min after the start of the glucose infusion during the IVGTT. The follow-up survey, at age 70 years, was carried out between 1991 and 1995. By January 1991, of the original cohort of 2,322 men, 422 had died and 219 had moved from the Uppsala region, leaving 1,681 men eligible for study. These men were invited to the third survey, and 1,221 men participated; of these, 1,219 underwent a standard oral glucose tolerance test according to 1985 World Health Organization criteria. Plasma glucose and

insulin concentrations were measured immediately before and 30, 60, 90, and 120 min after the glucose load. Of the 1,190 subjects available for genotyping in this study, 173 subjects, including the 7 subjects (4%) who already had diabetes at the time of inclusion, developed type 2 diabetes during the 20 years of follow-up. The follow-up survey at 70 years of age also included a 120-min hyperinsulinemic-euglycemic clamp ($56 \text{ mU} \cdot \text{min}^{-1} \cdot \text{m}^{-2}$) for estimating insulin sensitivity by measuring the IMGU (19). To evaluate to what degree hepatic glucose production was inhibited during the last hour in the present clamp protocol, we evaluated representatives from each of the following groups: 1) normal-weight control subjects ($n = 2$, BMI $24 \pm 1.5 \text{ kg/m}^2$, fasting serum insulin $5.1 \pm 3.4 \text{ mU/l}$), 2) obese normoinsulinemic subjects ($n = 2$, BMI $30.1 \pm 2.5 \text{ kg/m}^2$, fasting serum insulin $7.3 \pm 2.5 \text{ mU/l}$), 3) obese hyperinsulinemic subjects ($n = 2$, BMI $31.7 \pm 4.6 \text{ kg/m}^2$, fasting serum insulin $23.6 \pm 4.8 \text{ mU/l}$), and 4) obese type 2 diabetic subjects ($n = 3$, BMI $30.4 \pm 2.6 \text{ kg/m}^2$, fasting serum insulin $19.1 \pm 3.8 \text{ mU/l}$), were studied with glucose tracers in the infusate. Hepatic glucose output was suppressed by 95, 94, and 92%, in groups 1–3, respectively, and by 88% in the obese type 2 diabetic group (20). However, because we do not know the degree of hepatic glucose inhibition in all of the subjects, the IMGU is adjusted for the individual steady-state levels of insulin during the clamp (Table 1). Plasma glucose was measured by the glucose dehydrogenase method (Merck, Darmstadt, Germany), and the serum insulin concentration was measured by the Phadebas insulin test (Pharmacia AB, Uppsala, Sweden). The study was approved by the ethics committee of the Faculty of Medicine at the University of Uppsala, Sweden. Informed consent was obtained from all participants.

Genetic analyses. Genotyping for the Met326Ile variant of the p85 α was performed on genomic DNA isolated from blood cells, as previously described (12).

In vitro experiments. Construction of the Ile326 variant was performed by site-directed mutagenesis of full-length p85 α in a cytomegalovirus (CMV)-driven expression vector using QuickChange (Stratagene) and control sequencing with Dye Terminator and ABI Prism 377 Automatic Sequenator (Perkin Elmer), according to manufacturer's recommendations. Transfection of human embryonic kidney cells (HEK293) with human PKB α /CMV5 plasmids (0.5 $\mu\text{g/ml}$) and p85 α variants (0.1–0.5 $\mu\text{g/ml}$) were performed by the calcium phosphate method (16). After insulin (500 nmol/l) stimulation (0–30 min), cells were lysed; from lysate corresponding to 100 μg total protein, HA-PKB was immunoprecipitated at 4°C for 75 min with 12CA5 monoclonal anti-HA antibody followed by measurement of kinase activity in vitro, estimating the incorporation into the "Crosstide" of ^{32}P as described previously (16). Immunoblotting at room temperature, with 12CA5 anti-HA (1:200) and anti-p85 α (1:3,000) antibodies as primary antibodies and alkaline phosphatase-coupled anti-mouse (1:1,000) or anti-rabbit (1:2,000) antibodies as secondary antibodies, was performed according to standard procedures (16). Anti-phospho-Thr308/PKB antibodies (New England Biolabs) were used to detect phosphorylation of PKB at the Thr308.

Statistics. Skewed variables were log-transformed to reach normal distribution ($w > 0.95$), tested with Shapiro-Wilk's test. Normally distributed variables were used in all statistical analyses. For comparison of genotype groups, an unpaired t test was used for continuous variables. An analysis of covariance model was used to adjust for covariate variables, and because the study population was born in different geographical regions of Sweden, we adjusted for potential regional founder effects (relatedness) by including a covariate that reflected the genotypic distribution of the Met326Ile variant among the subjects born in the municipality of Uppsala or in other geographical regions of Sweden. However, including this covariate in the analyses did not add anything to the model. For categorical variables, Fisher's exact test was used. Statistical analyses were performed using SAS version 6.12 for Windows (SAS Institute, Cary, NC). All tests were two-tailed, and $P < 0.05$ was considered significant.

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