Hexokinase II-deficient Mice

PRENATAL DEATH OF HOMOZYGOATES WITHOUT DISTURBANCES IN GLUCOSE TOLERANCE IN HETEROZYGOTES*

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Type 2 diabetes is characterized by decreased rates of insulin-stimulated glucose uptake and utilization, reduced hexokinase II mRNA and enzyme production, and low basal levels of glucose 6-phosphate in insulin-sensitive skeletal muscle and adipose tissues. Hexokinase II is primarily expressed in muscle and adipose tissues where it catalyzes the phosphorylation of glucose to glucose 6-phosphate, a possible rate-limiting step for glucose disposal. To investigate the role of hexokinase II in insulin action and in glucose homeostasis as well as in mouse development, we generated a hexokinase II knock-out mouse. Mice homozygous for hexokinase II deficiency (HKII<sup>−/−</sup>) died at approximately 7.5 days post-fertilization, indicating that hexokinase II is vital for mouse embryogenesis after implantation and before organogenesis. HKII<sup>+/−</sup> mice were viable, fertile, and grew normally. Surprisingly, even though HKII<sup>+/−</sup> mice had significantly reduced (by 50%) hexokinase II mRNA and activity levels in skeletal muscle, heart, and adipose tissue, they did not exhibit impaired insulin action or glucose tolerance even when challenged with a high-fat diet.

Type 2 diabetes is a common disorder affecting about 3–5% of Western populations. Although the etiology of Type 2 diabetes has remained largely unknown, impaired insulin action in peripheral tissues coupled with a defect in insulin secretion capacity of the pancreatic β cells play a major role in the pathogenesis of this disease (1).

Type 2 diabetes is characterized by decreased rates of insulin-stimulated glucose uptake and utilization (2). The causes for impaired insulin action can be multiple, ranging from defects in insulin's binding to its receptor to the final steps of insulin's action on carbohydrate metabolism. Mutations in the insulin and insulin receptor genes are uncommon causes for insulin resistance (3, 4). The major site of impaired insulin action in Type 2 diabetes locates at the post-receptor level and remains to be determined. Insulin binding to its receptor activates a cascade of intracellular events. Tyrosine phosphorylation of the β-subunit of the insulin receptor activates insulin receptor substrates which mediate multiple insulin actions (5). The first step in glucose metabolism is its transport into cells where it is rapidly phosphorylated to glucose 6-phosphate (Glu-6-P) by hexokinases.

In mammalian tissues the phosphorylation of glucose to Glu-6-P is catalyzed by the four members of an isoenzyme family of hexokinases (ATP:β-hexose-6-phosphotransferase, EC 2.7.1.1) (6). Hexokinases I-III (100 kDa) exhibit high affinity to glucose and are subject to feedback inhibition by Glu-6-P. Glucokinase (50 kDa) exhibits low affinity to glucose and is not inhibited by physiological concentrations of Glu-6-P. It has been suggested that hexokinases I-III arose from an ancestral gene similar to glucokinase or yeast hexokinase by gene duplication and tandem ligation (7, 8). A defect in phosphorylation of glucose by the glucokinase gene (= hexokinase IV) results in impaired insulin secretion and maturity onset diabetes of the young (9, 10).

Glucose transport is commonly viewed as the rate-limiting step for insulin-mediated glucose metabolism in skeletal muscle (11). However, glucose phosphorylation could be a rate-limiting step under hyperglycemia (12). Indeed, reduced hexokinase II mRNA and enzyme production and low basal levels of Glu-6-P in skeletal muscle and adipose tissues, where hexokinase II is mainly expressed (13), have been found in Type 2 diabetic patients (14, 15). Hence, hexokinase II is a promising candidate gene for insulin resistance and Type 2 diabetes. Indeed, mutations in the coding region of hexokinase II gene have been observed in approximately 6% of Finnish Type 2 diabetic patients (16). The generation of mice lacking hexokinase II can give a definite answer as to whether or not diminished levels of hexokinase II can cause Type 2 diabetes or insulin resistance.

Previous studies, where mice homozygous for the null allele of glucose-6-phosphate isomerase (Glu-6-P isomerase) were embryonic lethal, have indicated that anaerobic glycolysis is the predominant energy delivering pathway in early post-implantation embryos (17, 18). Therefore, it is possible that glycolysis is critical for normal gastrulation in the mouse. Because hexokinase II regulates the key enzyme of glycolysis, targeted disruption of this gene provides information also on critical steps in mouse embryonic development.

In this paper we report the generation of a hexokinase II-deficient mouse. We demonstrate that, whereas homozygosity for the disrupted hexokinase II gene causes embryonic lethal-

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‡ The abbreviations used are: Glu-6-P, glucose-6-phosphate; Glu-6-P isomerase, glucose-6-phosphate isomerase; neo, neomycin phosphotransferase; IRS, insulin receptor substrate; kb, kilobase(s); PCR, polymerase chain reaction; ES, embryonic stem.

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ity, heterozygosity is not associated with abnormalities in glucose tolerance or insulin action.

MATERIALS AND METHODS

Animals—Animals were kept at the National Laboratory Animal Center of the University of Kuopio on a 12-h day/night cycle and were fed regular chow. Housing and procedures involving experimental animals were performed in accordance with protocols approved by the Animal Welfare and Use Committee of the University of Kuopio, Finland.

Construction of the Targeting Vector—To clone the mouse hexokinase II gene, 129/SvJ mouse genomic library (Stratagene Inc., La Jolla, CA) was screened with rat hexokinase II cDNA (gift from Dr. J. E. Wilson, Michigan State University). The region of hexokinase II enzyme encoded by exon 4 and onwards has been shown to contain glucose and ATP-binding sites (6). Two DNA sequences containing mouse hexokinase II exon 4 (XhoI fragment) and exons 5–10 (XhoI-XhoI fragment) were subcloned to pUC19 cloning vector, yielding p8X4 and p2XS1, respectively. The mouse hexokinase II gene was disrupted by inserting a positive selection cassette from pTV-0 (gift from Dr. H. van der Grinten, CA) essentially as described previously (20). To screen for the correctly targeted hexokinase II gene, the cell clones surviving neomycin selection were analyzed by PCR under standard conditions using a forward primer within the 5′-region outside of the targeting vector (HK2KO-F1, 5′-AACCGACGGCCCAATGATTAG-3′) and a reverse primer within the neo cassette (Neo-R1, 5′-GTCGCCAGTCTACGCGAATAGCT-3′), as indicated in Fig. 1A. To verify the correct targeting, 10 μg aliquots of genomic DNA from PCR-positive clones were digested either with XbaI or BamHI and subjected to Southern blot analysis using the PstI-XhoI (Fig. 1A, probe a) or XhoI-VPxI (Fig. 1A, probe b) fragments of the mouse HKII gene, respectively, as probes. Blots of XhoI-digested DNA were also probed for neo (Fig. 1A, probe c) in order to verify the lack of additional random integration of the targeting vector.

Generation of Chimeric Mice and Germline Transmission of the Disrupted Allele—ES cells carrying the correctly disrupted hexokinase II gene were aggregated with BALB/c × DBA/2 morulae (21). Morula aggregates were transferred into pseudopregnant BALB/c × DBA/2 foster mothers. The resulting chimeric mice, identified by the presence of agouti coat pigmentation, were bred to BALB/c × DBA/2 females to establish germline transmission of the disrupted allele. The offspring carrying disrupted hexokinase II alleles was identified by PCR as described for ES cells. Further verification of correct targeting was accomplished by PCR amplification with forward primer HK2KO-F2 (5′-ACTCTCTTGCCGCTGGCCG-3′) and reverse primers Neo-R1 and HK2KO-R1 (Fig. 1A). This allele discriminating PCR scheme allowed convenient detection of both wild type and disrupted hexokinase II alleles in one PCR reaction. The established mouse lines carrying the disrupted hexokinase II allele were identified as Ukko1, -2, and -3. HKII alleles were further backcrossed to BALB/c × DBA/2 females to dilute the 129/Ola genetic background originating from the embryonic stem cells. An attempt was also made to breed the chimeras directly with the 129/Ola mouse line (Harlan UK Limited, Blackthorn, UK), but this proved to be virtually impossible due to the very poor reproduction capacity of 129/Ola mice.

Characterization of Embryonic Lethality—HKII-/- mice were mated and offspring was analyzed by allele discriminating PCR, and by Southern blotting, as described above. Since no HKII-/- offspring was detected, the embryos at selected developmental stages were analyzed. To analyze blastocysts, morulae were flushed from the oviducts at the third day of the pregnancy and grown in M16 medium overnight. Blastocysts detected visually as viable were washed in 1% phosphate-buffered saline supplemented with 1 mg/ml bovine serum albumin and transferred individually to 3 μl of 1 × PCR buffer containing 50 μg/ml protease K, lysed for 2 h at 55 °C, and subjected to allele discriminating PCR as described above. To analyze gastrulae, embryos were dissected at 7.5 and 8.5 days of gestation, cultured overnight in 1 × phosphate-buffered saline, photographed, and analyzed by allele discriminating PCR as described above.

Northern Blot Analysis of Hexokinase II mRNA Expression—Total RNA was isolated from hind limb muscle, heart, and adipose tissue of 10-week-old HKII-/- and HKII-/- male Ukko1F2 mice by the acid guanidinium thiocyanate method (22). Fifteen to 20 μg of total RNA were fractionated by electrophoresis in 1.2% agarose gel under denaturing conditions, transferred onto positively charged nylon membrane, and hybridized with a digoxigenin-labeled mouse hexokinase II cDNA probe encompassing exons 11 to 13. The blots were visualized on autoradiography film, and hexokinase II mRNA expression levels were determined from scanned images of the blots. Equal loading of RNAs was verified by methylene blue staining of the membrane.

Assay of Hexokinase II Activity—HKII-/- and HKII-/- male Ukko1F2 hind limb muscle, heart, and adipose tissue, 50–300 mg each, were homogenized on ice for 45 s with Teflon pestle in 4–5 volumes of TEN buffer (25 mm Tris-HCl, pH 8.0, 10 mm EDTA, 150 mm NaCl) supplemented with 10 mm monothioglycerol. Homogenates were sonicated on ice and subjected to 13,000 rpm centrifugation at +4 °C. One aliquot of the soluble fraction was used to assay total hexokinase activity, and another was heat-treated at +45 °C for 1 h and assayed for the heat stable hexokinase activity, mainly hexokinase I (23, 24). Hexokinase activity was determined at room temperature by measuring the formation of coenzyme NADPH in a coupled enzyme assay system containing Glu-6-P dehydrogenase from Leucostoc mesenteroides (Sigma). Protein concentrations were determined using the Bio-Rad protein assay kit with bovine serum albumin as a standard. Enzyme activities for hexokinase I and II are expressed as milliunits per minute per milligram protein, where 1 unit equals to the amount of enzyme activity forming 1 μM NADPH in 1 min at 23 °C and pH 8.0.

Plasma Glucose and Insulin Tolerance Tests—For the insulin tolerance test, 5 HKII-/- and 5 HKII-/- female, 16-week-old Ukko1F2 mice were fasted for more than 12 h, anesthetized with fentanyl-fluanisone (Janssen Pharmaceutica, Beerse, Belgium) and midazolam (Hoffmann-LaRoche, Basel, Switzerland), and placed on a warm (37 °C) surface. After intraperitoneal injection of 0.75 milliunits/g (body weight) insulin (Actrapid, Novo Nordisk, Denmark), samples of blood were drawn at different time points (0, 15, 30, and 60 min) from the tail vein. Plasma glucose concentrations were determined by glucose & lactate analyzer 2300 Stat Plus (YSI, Yellow Springs, OH).

For the glucose tolerance test, 10 HKII-/- and 10 HKII-/- male, 11-week-old Ukko1F2 mice were prepared as described above, and intraperitoneally injected with 1 mg/g (body weight) g-glucose. Blood samples were drawn at different time points (0, 15, 30, and 60 min) from the tail vein. Plasma glucose levels were determined using a rat insulin ELISA kit (Crystal Chem Inc., Chicago, IL) with mouse insulin as a standard. Plasma glucose concentrations were determined as described above.

Feeding with High-fat Diet—Six HKII-/-, 5 HKII-/- male and 5 HKII-/- female Ukko1F2 mice, were fed from the age of 4 weeks onwards a Western type diet (Harlan Teklad, Madison, WI), where 42% of the calories were from fat. Four HKII-/-, 4 HKII-/- male and 5 HKII-/-, 4 HKII-/- female littersmates were fed a regular low-fat diet (4% fat). At 0, 4, and 8 weeks of feeding the mice were weighed and blood samples were drawn from the saphenous vein (25) after a 4–6 h fast (H2O ad libitum). Plasma glucose and insulin concentrations were measured as described above. After 14 weeks, insulin tolerance test was performed as described above, except that a 4-h fast was used. Blood was drawn from the saphenous vein and blood glucose concentrations were determined by Precision Q.L®TM blood glucose sensor with Precision Plus electrodes (MediSense, Abbott Laboratories Ltd., Birmingham, UK). Fasting plasma from the first time point was also assayed for the triglyceride and total cholesterol concentrations by kits purchased from Roche Molecular Biochemicals. After the last sample was collected the mice were intraperitoneally injected with approximately 0.5 mg/g (body weight) g-glucose and allowed body weight. After 16 weeks to recover. A glucose tolerance test was performed as described above, except that a 4-h fast was used and blood was collected from the saphenous vein.

Statistical Analysis—Calculations were performed with the SPSS for Windows programs (SPSS Inc., Chicago, IL). Data are shown as mean ± S.E. Statistical analyses of data were performed with Student's t test for independent samples, Chi-square, or ANOVA, when appropriate. Glu-
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**Fig. 1. Disruption of the mouse hexokinase II gene by homologous recombination.** A, schematically presented structures of the wild type hexokinase II allele, targeting vector pHK2Neo, and the disrupted hexokinase II allele. Exons (black boxes) and selected restriction sites (B, BamHI; P, PstI; V, VspI; X, XbaI, and X, XhoI) are indicated. Targeting vector contains a gene for neomycin phosphotransferase (neo) to allow the selection of homologous recombination events and to disrupt the coding region of the hexokinase II gene in exon 4. The 10.7-kb VspI fragment electroporated into embryonic stem cells is indicated under the targeting vector. Southern analysis probes (black bars) a and b are 2.0-kb PstI-XbaI and 0.7-kb XbaI-VspI genomic fragments, respectively, located upstream of the 5' homology arm of the targeting vector. Probe c is a 1.2-kb fragment containing the neo expression cassette. Wild type and disrupted genomic BamHI fragments (11.5 and 7.8 kb, respectively), and XbaI-fragments (2.2 and 3.3 kb, respectively) recognized by probes a, b, and/or c are indicated. PCR primers for identification of the embryonic stem cell clones with targeted hexokinase II allele were HK2KO-F1 (open forward arrowhead) and Neo-R1 (open reverse arrowhead). The embryos and the mice were genotyped by allele discriminating PCR with primers HK2KO-F2 (solid forward arrowhead), Neo-R1, and HK2KO-R1 (solid reverse arrowhead). B, Southern analysis of neomycin selected, PCR positive embryonic stem cell clones, and offspring of the established hexokinase II-deficient mouse line. Genomic BamHI-digested DNA was hybridized with intronic probe a described above. Expected banding pattern was observed on clones ES9 and ES10 (lanes 1 and 2) from the mutant mouse lines Ukk01 and Ukk02, respectively, originated and on HKII+/− and HKII−/− Ukk01F2 female offspring (lanes 3 and 4). C, allele discriminating PCR used for genotyping of the embryos and post-partum mice. Primers used are described above. From the left are shown products from the PCRs of HKII+/− (239 base pairs), HKII−/− (239 and 432 base pairs), and HKII−/− (432 base pairs) blastocysts. In both B and C, the molecular weight marker as kb is indicated on the right.

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Hexokinase II-deficient Mice—The mouse hexokinase II gene was inactivated using a replacement type targeting vector containing 9.8 kb of isogenic DNA in which the neo gene was inserted into exon 4 of the mouse hexokinase II gene (Fig. 1A). The targeting construct was electroporated into E14 embryonic stem cells (genetic background 129/Ola). 138 stable neomycin-resistant clones were first analyzed by PCR with primers HK2KO-F1 and Neo-R1 (Fig. 1A). To verify the correct targeting of the hexokinase II gene, the PCR-positive clones were further analyzed by Southern blotting of genomic DNA using probes a or b, and c (Fig. 1A). Nine clones displayed the expected DNA restriction fragments without any evidence for additional integrations of the targeting construct. A Southern blot of BamHI-digested embryonic stem cell DNA probed with probe a is presented in Fig. 1B. Cells from three correctly targeted clones (ES4, ES9, and ES10) were aggregated with BALB/c × DBA/2 morulae. Aggregated chimeric morulae were placed into the oviducts of pseudopregnant BALB/c × DBA/2 females resulting in a total of 11 mice with chimeric coat color pattern. Upon mating to BALB/c × DBA/2 mice, germline transmission was achieved with three chimeric (20%, ±100%) male mice derived from two different mutant ES clones (ES9 and ES10). The near 100% chimera derived from ES10 was somewhat retarded, achieving fertility as late as at 20 weeks of age. This line (Ukk03) was not studied further. Both Ukk01 and 2 lines, derived from mutat ES9 and ES10 clones, respectively, were used for observations on viability, fertility, and growth. For further characterization of biochemical and metabolic consequences of the hexokinase II-deficiency, only the Ukk01 line, originating from clone ES9, was used.

**Viability, Fertility, and Growth of HKII-deficient Mice—** Both male and female HKII−/− mice were fertile and produced viable offspring with BALB/c × DBA/2 at approximately 1:1 ratio of females to males. However, the matings of HKII+/− males with HKII+/− females produced no HKII−/− offspring. Of the 64 born mice, 21 were HKII+/− (32.8%) and 43 were HKII−/− (67.2%), but no HKII−/− mice were found. Hence the HKII−/− genotype was assumed to be embryonic lethal. This was confirmed by the nonsignificant difference (p = 0.934) between the observed HKII+/−:HKII−/− ratio and the expected 1:2 ratio in the Chi-square test.

To compare the growth of HKII+/− and HKII−/− mice, they were first weighed at weaning (3 weeks of age) and at 1–2 week intervals for up to 6 months. Among the progeny of the first backcross (BALB/c × DBA/2), male, but not female, HKII+/− mice (n = 8 or 15 per time point) weighed less than HKII−/− littermates (n = 7 or 15) (p = 0.12 to p < 0.01). Further backcrosses to BALB/c × DBA/2 were made to dilute the embryonic stem cell-derived genetic background (129/Ola). Among the progeny of the third backcross, where approximately 90% of the 129/Ola genome is removed, no weight difference was observed between HKII−/− and HKII+/− mice of either sex between weaning and 27 weeks of age. In addition, growth in utero was not affected by heterozygous hexokinase II deficiency, since the body weight of both female and male newborn offspring of F1 backcross parents were similar: 1.35 ± 0.03 g (n = 21) for HKII+/− females versus 1.32 ± 0.03 g (n = 17) for HKII−/− females (p = 0.49), and 1.36 ± 0.05 g (n = 16) for HKII+/− males versus 1.31 ± 0.04 g (n = 18) for HKII−/− males (p = 0.42).

**Staging the Prenatal Death of HKII−/− Embryos—** As an initial step to investigate the stage of prenatal death, morulae were flushed 2.5 days post-coitum from the oviducts of pregnant HKII+/− females (mated with HKII−/− males), and grown in culture medium overnight. Resulting viable blastocysts were genotyped as HKII+/− (21.2%), HKII−/− (51.5%), and HKII−/− (27.3%) (ES5 in Table I). A typical PCR result is shown in Fig. 1C. Viable blastocysts showed a Mendelian distribution of wild type and targeted genotypes by the Chi-square test (p = 0.873).

Since previous studies on Glu-6-P isomerase-deficient mice have suggested that functional glycolysis is vital for mouse gastrulation (17, 18), this stage was investigated next. Embryos were dissected 7.5 and 8.5 days post-coitum from the uteri of pregnant HKII−/− females mated with HKII−/− males...
and genotyped. Among the 32 E7.5 embryos, 28.1% were HKII+/−, 50.0% were HKII+/+, and 21.9% were HKII−/− (Table I). However, 3 out of 7 HKII−/− embryos at this stage were visibly retarded in size. Among the 19 E8.5 embryos, all 5 HKII−/− embryos were severely retarded in growth. Three of them are shown in Fig. 2 along with an HKII+/− E8.5 embryo that had developed normally.

Hexokinase II mRNA and Enzyme Activity Assays—Hexokinase II mRNA levels of 5 HKII+/− and 4 HKII−/− male Ukko1F2 mice were assayed by Northern analysis in insulin-responsive skeletal muscle, heart, and adipose tissue, previously demonstrated to be the main hexokinase II expressing mammalian tissues. Expression levels were determined from scanned autoradiographs of the blots. The steady state level of hexokinase II mRNA expression in tissues of HKII−/− mice was clearly lower (adipose, 41.1%, heart, 49.6%, and muscle 37.9% of the wild type hexokinase II expression) than in the HKII+/− littermates (Fig. 3).

Next, hexokinase II enzyme activity was determined in the soluble fraction of the skeletal muscle, heart, and adipose tissue of 5 HKII+/− and 4 HKII−/− male Ukko1F2 mice. The total and heat stable hexokinase activity were measured by the NADPH-coupled assay. As described previously (24), hexokinase I is mainly responsible for the heat stable activity while hexokinase II is mostly inactivated by heat (1 h at 45 °C). Thus, the heat stable activity represents hexokinase I activity in tissues whereas total minus heat stable hexokinase activity represents hexokinase II activity. This was recently verified for the soluble fraction of the heart tissue by Wilson (23), although the heat stability of hexokinase I was shown to vary substantially depending on the tissue and subcellular fraction used. Enzyme activities of hexokinase I and II of HKII−/− mice were compared with activities of HKII+/− littermates, and all tissues studied showed significantly reduced hexokinase II activity (adipose, 53.3%, heart, 44.0%, and muscle 49.5% of the wild type hexokinase II activity) but unaltered hexokinase I activity (adipose, 118.6%, heart, 79.2%, and muscle 111.7% of the wild type hexokinase I activity) (Fig. 4).

Insulin and Glucose Tolerance Tests—To determine whether the approximate 50% loss of hexokinase II activity in insulin-sensitive tissues results in insulin resistance or diabetes, mice were subjected to insulin and glucose tolerance tests. In the insulin tolerance test, 0.75 milligrams/g of insulin was given intraperitoneally to 16-week-old weight-matched fasted female Ukko1F2 littermates (5 HKII+/− and 5 HKII−/−). The glucose lowering effect of insulin was determined at 0, 15, 30, and 60 min after insulin injection. Glucose values did not differ significantly between the HKII+/+ and HKII−/− mice (Fig. 5A).

The glucose tolerance test was performed with fasted Ukko1F2 male mice (10 HKII+/+ and 10 HKII−/−). Intraperitoneal injection of 1 mg/g of glucose was administered and the levels of glucose and insulin were determined at 0, 15, 30, and 60 min after glucose injection. After correction for differences in fasting weight (p < 0.05), the groups had similar glucose and insulin values, although at 60 min HKII−/− mice had significantly lower glucose and insulin (p < 0.05) levels compared with those of HKII+/+ mice (Fig. 5B).

Effects of High-fat Feeding on Insulin Resistance and Glucose Tolerance—To verify the lack of association between reduced hexokinase II activity and abnormalities in insulin action or glucose tolerance, the mice were challenged with high-fat diet to induce insulin resistance. After 14 weeks of feeding a high-fat diet, HKII+/+ and HKII−/− mice had over 2-fold higher total plasma cholesterol concentrations (4.37 ± 0.35 and 4.11 ± 0.24 mm, respectively) than those fed a low-fat diet (1.88 ± 0.13 and 2.03 ± 0.22 mm, respectively) (p = not significant between the genotypes). Plasma triglycerides did not change significantly. Feeding a high-fat diet increased body weight (Table III), although the difference in body weight between the diets was not statistically significant. In addition, the feeding with high-fat diet raised plasma insulin concentrations of HKII+/− mice after 16 weeks (p < 0.05) and of HKII−/− mice after 4, 8, and 16 weeks (p < 0.01 each) indicating the induction of insulin resistance in both groups (Table II). However, no statistically significant differences in insulin levels were observed between HKII+/+ and HKII−/− mice, either on low-fat or high-fat diet. Insulin tolerance test showed a tendency toward insulin resistance in mice fed with high-fat diet without significant differences between HKII+/+ and HKII−/− mice (Fig. 6A). Similarly, the glucose tolerance test (Fig. 6B) did not reveal differences in plasma glucose and insulin concentrations between HKII+/+ and HKII−/− mice. Both HKII+/+ and HKII−/− mice fed a high-fat diet had increased insulin levels at 0, 15, and 30 min after glucose injection compared with corresponding insulin levels of HKII+/+ and HKII−/− mice fed a low-fat diet. Glucose concentration at 60 min was higher on high-fat feeding than on low-fat feeding (p = 0.076 for HKII+/+ mice and p < 0.05 for HKII−/− mice).

Discussion

We have generated a mouse line in which the hexokinase II gene was disrupted by homologous recombination. The following questions were addressed. 1) Does the deficiency of hexokinase II lead to the development of insulin resistance and Type 2 diabetes? 2) Is hexokinase II essential for normal embryonic development?

PCR amplification and Southern blot analyses provided unequivocal evidence for correct targeting of the hexokinase II gene. Homozygous hexokinase II knock-out mice were embryonic lethal at approximately the E7.5 stage. Heterozygotes for the disrupted gene were viable and exhibited approximately 50% of the normal levels of hexokinase II mRNA and activity in adipose, heart, and skeletal muscle.

Embryonic death of the HKII−/− mice points to a vital role of hexokinase II after the blastocyst stage but before organogenesis, most likely at early gastrulation. Embryonic lethality was also observed in Glu-6-P isomerase-deficient mice, indicating that glycolysis is necessary for early embryonic development (17, 18, 26). Mouse embryos that were completely deficient in Glu-6-P isomerase failed to develop beyond the egg cylinder stage and gastrulation was not completed due to failure to produce a functional mesoderm. Mice with 20% Glu-6-P isomerase activity were viable but had hemolytic anemia and elevated plasma glucose concentrations whereas those having 60% activity had unaltered hematological and physiological parameters (27). A defect in hexokinase activity immediately upstream of Glu-6-P isomerase in the glycolytic pathway would

| Age of embryos or mice | HKII+/+ | HKII−/− | HKII+/− |
|------------------------|---------|---------|---------|
| E3.5                   | 7 (21.2%) | 17 (51.5%) | 9 (27.3%) |
| E7.5                   | 9 (28.1%) | 16 (50.0%) | 7 (21.9%) |
| E8.5                   | 6 (31.6%) | 8 (42.1%) | 5 (26.3%) |
| D21                    | 21 (32.8%) | 43 (67.2%) | 0 (0%) |

* Three out of the 7 E7.5 HKII−/− embryos were retarded in growth.
* All of the E8.5 HKII−/− embryos were severely retarded in growth.

| TABLE I

Distribution of hexokinase II genotypes of Ukko1F2 littermates at different stages of development

HKII+/− mice were mated and resulting embryos (E) or post partum mice (D) were genotyped at indicated time points (days) with allele discriminating PCR as described under “Materials and Methods.” Data are presented as a number of embryos followed by percentage in parentheses.
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**Fig. 2.** Growth retardation of HKII<sup>−/−</sup> embryos at 8.5 days of development. Embryos resulting from the matings of HKII<sup>−/−</sup> mice were removed from pregnant mothers at various time points to determine the temporal window of prenatal death of HKII<sup>−/−</sup> embryos. The figure shows a normally developed E8.5 HKII<sup>−/−</sup> embryo (+/−) and three retarded E8.5 HKII<sup>−/−</sup> embryos (−/−) from the same mother. Magnification is 215-fold.

**Fig. 3.** Northern analysis of hexokinase II mRNA expression in insulin-sensitive tissues. Total RNA was isolated from adipose tissue, heart, and hind limb muscle of 5 HKII<sup>−/−</sup> (+/+), 4 HKII<sup>−/−</sup> (+/−) and 4 HKII<sup>−/−</sup> (−/−) mice. Total liver RNA was included as negative control (−/−). Northern hybridization was performed with a probe encompassing exons 11 to 13 of the mouse hexokinase II coding sequence. Equal loading of RNAs was verified by methylene blue staining of the membrane (not shown).

**Fig. 4.** Enzyme activities of hexokinase I and II in insulin-sensitive tissues. Tissue samples from adipose, heart, and hind limb muscle from 5 HKII<sup>−/−</sup> (solid bars) and 4 HKII<sup>−/−</sup> (open bars) mice were homogenized and enzyme activities were measured using NADPH coupled assay. Data are presented as mean percent of wild type hexokinase II activity for each tissue ± S.E. The significant differences between the groups are indicated as: *, p < 0.05, and ***, p < 0.001.

likely result in similar or even more extensive defects, since the pentose phosphate shunt, which is functional even in the absence of Glu-6-P isomerase, would be completely blocked in cells devoid of hexokinase II activity. On the other hand, since hexokinase II is only one of four hexokinases, one would expect that the phosphorylation of glucose to Glu-6-P could be compensated by other hexokinases. Indeed, the proteins or mRNAs of different hexokinases have been detected in rat embryonic tissues at the later stages of development (e.g., 28–31), but the synthesis patterns of hexokinases have not yet been determined during the early post-implantation stage. The obvious lack of significant compensation by other hexokinases demonstrated by the in utero death of HKII<sup>−/−</sup> embryos is surprising also, since mice deficient of another hexokinase, namely glucokinase, survive to term (32, 33). Thus, our results suggest a vital and unique role for hexokinase II in mouse development.

The application of knock-out technology in vivo has provided new models to evaluate the role of specific genes in the etiology of Type 2 diabetes. Knock-out mice for genes regulating insulin secretion or insulin action have been generated. Homozygous mice carrying a null mutation in the glucokinase gene generated by disrupting the pancreatic β-cell specific exon exhibited severe diabetes shortly after birth and died within a week, whereas heterozygotes showed early-onset mild diabetes due to impaired insulin secretory response to glucose (32, 33). Several mouse knock-out models for insulin action have also been generated. Homozygous mice for insulin receptor knock-out died shortly after birth with severe hyperglycemia. In contrast, heterozygous mice compensated for insulin resistance and had normal glucose tolerance (34, 35). Recently Lauro et al. (36) generated transgenic mice with insulin resistance in muscle and adipose tissue by crossing mice heterozygous for a targeted null allele of the insulin receptor gene with transgenic mice expressing a kinase-defective human insulin receptor. Although these mice developed peripheral insulin resistance they failed to become diabetic. Insulin receptor substrate 1 (IRS-1) knock-out mice exhibited insulin resistance and glucose intolerance but did not have frank diabetes (37, 38). In contrast to IRS-1, the disruption of IRS-2 impairs both peripheral insulin signaling and pancreatic β-cell function (39). These mice show progressive deterioration of glucose homeostasis because of insulin resistance in the liver and skeletal muscle and a lack of
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The HKII\(^{-/-}\) and HKII\(^{+/+}\) mice were fed for 16 weeks, starting at 4 weeks of age, with a high-fat or low-fat diet and their fasting weight, plasma glucose, and insulin levels were determined as described under “Materials and Methods.” Data are presented as mean ± S.E. No statistically significant differences were observed between HKII\(^{-/-}\) and HKII\(^{+/+}\) mice at any time point during low-fat or high-fat feeding.

**TABLE II**

Effect of feeding with low-fat and high-fat diet

| Genotype | Low-fat diet | High-fat diet |
|----------|--------------|---------------|
|          | 0 wk | 4 wk | 8 wk | 16 wk | 0 wk | 4 wk | 8 wk | 16 wk |
| Animals (n) | HKII\(^{+/+}\) | 9 | 9 | 9 | 9 | 11 | 11 | 11 | 11 |
|            | HKII\(^{-/-}\) | 8 | 8 | 8 | 8 | 11 | 11 | 11 | 11 |
| Weight (g) | HKII\(^{+/+}\) | 12.3 ± 0.3 | 19.4 ± 0.8 | 23.1 ± 1.1 | 26.6 ± 1.7 | 11.2 ± 0.5 | 19.9 ± 1.3 | 25.7 ± 1.9 | 29.6 ± 2.6 |
|            | HKII\(^{-/-}\) | 12.6 ± 0.6 | 19.1 ± 1.3 | 21.9 ± 1.5 | 25.7 ± 1.8 | 12.5 ± 0.5 | 21.3 ± 1.2 | 26.6 ± 1.9 | 31.5 ± 2.4 |
| Glucose (mM) | HKII\(^{+/+}\) | 6.1 ± 0.4 | 9.0 ± 0.4 | 7.9 ± 0.5 | 10.1 ± 1.8 | 6.0 ± 0.8 | 8.8 ± 0.5 | 9.5 ± 0.4\(a\) | 9.9 ± 0.9 |
|            | HKII\(^{-/-}\) | 5.5 ± 0.6 | 7.9 ± 0.6 | 6.7 ± 0.7 | 7.7 ± 0.7 | 5.9 ± 0.7 | 9.5 ± 0.4\(a\) | 9.6 ± 0.6\(b\) | 9.8 ± 0.9 |
| Insulin (ng/ml) | HKII\(^{+/+}\) | 0.19 ± 0.03 | 0.55 ± 0.11 | 0.76 ± 0.17 | 0.99 ± 0.23 | 0.29 ± 0.09 | 1.04 ± 0.31 | 1.38 ± 0.34 | 5.25 ± 1.49\(a\) |
|            | HKII\(^{-/-}\) | 0.22 ± 0.05 | 0.32 ± 0.04 | 0.41 ± 0.10 | 1.12 ± 0.16 | 0.25 ± 0.04 | 1.05 ± 0.19\(a\) | 1.47 ± 0.32\(b\) | 5.65 ± 1.34\(a\) |

\(a\) \(p < 0.05\) between the low-fat and high-fat diet at a given time point in each genotype (Student’s \(t\) test).

\(b\) \(p < 0.01\).

**FIG. 6.** Effect of low-fat and high-fat diet on glucose and insulin tolerance of HKII\(^{-/-}\) and HKII\(^{+/+}\) mice.  
A, insulin tolerance test (intraperitoneal, 0.75 milliuunits/g body weight insulin); and B, glucose tolerance test (intraperitoneal, 1 mg/kg body weight glucose) of HKII\(^{-/-}\) (○) and HKII\(^{+/+}\) (□) mice (8–11 animals per group) fed with low-fat (solid line) or high-fat (broken line) diet. Blood samples were collected from the saphenous vein at the indicated time points and analyzed for plasma glucose and insulin concentrations. Data in A are presented as mean percent of 0 min glucose value ± S.E., and in B as mean of glucose (mM) and insulin (ng/ml) values ± S.E. Statistically significant differences between the diets are indicated as: *, \(p < 0.05\) and **, \(p < 0.01\) for HKII\(^{-/-}\), and †, \(p < 0.05\) for HKII\(^{+/+}\) mice. Differences between genotypes in either diet were not statistically significant.

β-cell compensation for this insulin resistance. Double knock-out mice with disruption of IRS-1 and β-cell glucokinase developed overt diabetes (40). Similarly, double heterozygous mice for null alleles in insulin receptor and IRS-1 became overtly diabetic (41).

Because hexokinase II is the key enzyme in the glycolytic pathway, it is a potential candidate gene for insulin resistance. Hexokinase II is mainly expressed in skeletal muscle and adipose tissue (13) which are the most important sites for insulin-mediated glucose uptake. Since homozygous knock-out mice were embryonic lethal, we were able to investigate the degree of insulin resistance and glucose tolerance only in mice heterozygous for targeted disruption of the hexokinase II gene. Our results show that these mice were neither insulin-resistant nor glucose-intolerant even when fed a high-fat diet for up to 16 weeks. In fact, in intraperitoneal glucose tolerance test the HKII\(^{-/-}\) mice had lower plasma glucose and insulin at 60 min (Fig. 5B). These findings indicate that even 50% reduction of hexokinase II activity can be compensated without a significant impairment in insulin action or glucose tolerance. However, a slight impairment may remain uncovered due to the increased variation resulting from the still heterogenous genetic background of Ukk1P\(^{1+}\) mice, theoretically, 6.3% of 129/Ola and 46.9% of both BALB/c and DBA/2. Nevertheless, these results are consistent with two previous observations, first, that heterozygous Glu-6-P isomerase-deficient mice had normal glucose levels (18) and second, that transgenic mice over-expressing hexokinase II (3–8-fold) in striated muscle exhibited no changes in glucose tolerance or insulin action (12). In addition, overexpression of both the glucose transporter 4 and hexokinase II in double transgenic mice had no significant effect on glucose homeostasis (42). Whether or not a complete lack of hexokinase II would cause insulin resistance or glucose intolerance will have to be tested in a conditional hexokinase II knock-out mouse model.

Impaired insulin action to promote muscle glucose uptake is a characteristic finding of obesity and Type 2 diabetes. Abnormal glycogen synthesis has been often observed in insulin resistant states but simultaneous defects in glucose transport and/or glucose phosphorylation could also explain decreased rates of glucose uptake (11, 12). Saccomani et al. (43) demonstrated, by an isotopic tracer technique combined with mathematical modeling, that the defect in glucose phosphorylation (−80%) was greater than the defect in glucose transport (−40%). Recent studies (15, 44) also have shown that physiological hyperinsulinemia maximally stimulated hexokinase II mRNA in healthy subjects but the increase was significantly reduced in obese subjects and in patients with Type 2 diabetes. Thus, defect in hexokinase II activity could potentially contribute to insulin resistance. However, our results indicate that at least in animal model glucose phosphorylation cannot be a rate-limiting step for glucose metabolism if 50% of hexokinase II activity is preserved.

In conclusion, our study demonstrates that hexokinase II has a vital and unique role among the hexokinases in early mouse development, especially during gastrulation. Surprisingly, disruption of one allele of the hexokinase II gene did not lead to impaired insulin action or abnormal glucose tolerance, even when mice were challenged with a high fat diet.
