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SAMI HEIKKINEN

# Glucose Metabolism in Heterozygous Hexokinase II Knock-out Mice

Doctoral dissertation

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## ABSTRACT

Hexokinase (HK) II, which catalyzes the phosphorylation of glucose to glucose-6-phosphate in muscle and adipose tissue, has a central role in glucose metabolism. HKII has been implicated in type 2 diabetes since HKII expression is regulated by insulin, and has been found to be reduced in patients with type 2 diabetes. The aims of this study were to characterize the mouse HKII gene, generate a HKII knock-out mouse line, and investigate the role of HKII in mouse development and glucose metabolism.

The mouse HKII gene and full-length cDNA were cloned, sequenced and characterized, and shown to be closely related to previously characterized rat and human sequences. The tissue distribution of the mouse HKII mRNA expression was determined, and found to be similar to that for the rat and human. The mouse HKII gene promoter organization and mRNA transcription initiation and termination sites were also determined. Mouse HKII cDNA was cloned into a vector that can be used in expression studies.

A mouse HKII gene targeting vector was constructed and used to inactivate HKII in mouse embryonic stem cells. Subsequently, a HKII knock-out mouse line was generated. Homozygous HKII knock-out embryos died approximately at embryonic day 7. Heterozygous HKII knock-out ($HKII^{+/-}$) mice were viable, but had about a 50 % decrease in HKII mRNA and enzyme activity in the heart, skeletal muscle and adipose tissue. However, circulating levels of glucose, insulin, and other metabolites were not altered in $HKII^{+/-}$ mice in the basal state. Also glucose tolerance and insulin sensitivity of $HKII^{+/-}$ mice was similar to the wild type. In addition, insulin resistance associated with aging or a high-fat diet affected wild-type and $HKII^{+/-}$ mice equally.

The effect of moderate-intensity running exercise on the glucose metabolism of wild-type and $HKII^{+/-}$ mice was investigated. Exercise-stimulated glucose uptake was impaired in oxidative muscles (heart and soleus) of $HKII^{+/-}$ mice, whereas in glycolytic muscles (gastrocnemius and superficial vastus lateralis) the effect was apparently masked by increased exercise-stimulated glycogen breakdown. Glucose tolerance of $HKII^{+/-}$ mice was also mildly impaired during moderate-intensity exercise. However, the expression of several genes for key glycolytic enzymes and insulin signalling molecules was similar between wild-type and $HKII^{+/-}$ mice at the basal state and after exercise.

These results show that HKII has a vital and unique role in mouse development. They also confirm that glucose phosphorylation by HKII is not rate-limiting for glucose utilization in mice either in the basal state, during aging, or after high-fat feeding. However, glucose phosphorylation was shown to be rate-limiting for muscle glucose uptake and whole-body glucose tolerance during exercise.

National Library of Medicine Classification: QU 75, QU 141, QY 60.R6
Medical Subject Headings: hexokinase; glucose; phosphorylation; diabetes mellitus, type II; animals, genetically modified; mice, knockout; embryo and fetal development; glucose/metabolism; glucose/blood; insulin/blood; glucose tolerance test; glucose intolerance; aging; dietary fats; exercise/physiology; gene expression

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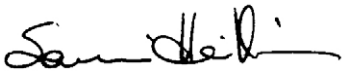
Kuopio, December 2003



Sami Heikkinen

## ABBREVIATIONS

| | |
|---|---|
| ADP | adenosine diphosphate |
| AICAR | 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside |
| AMP | adenosine monophosphate |
| AMPK | 5'-AMP-activated protein kinase |
| ANOVA | analysis of variance |
| ATF-1 | activating transcription factor 1 |
| AUC | area under the curve |
| BMI | body mass index |
| bp | base pair |
| cAMP | cyclic AMP |
| cDNA | complementary DNA |
| Chol | cholesterol |
| cM | centiMorgan |
| CRE | cAMP responsive element |
| CREB | CRE binding protein |
| CREM | CRE modulator |
| DG | deoxyglucose |
| DNA | deoxyribonucleic acid |
| ELISA | enzyme linked immunosorbent assay |
| EMBL | European Molecular Biology Laboratory |
| FFA | free fatty acid |
| G6P | glucose-6-phosphate |
| GAPDH | glyceraldehyde-3-phosphate dehydrogenase |
| GK | glucokinase |
| $GK^{\beta}$ | β-cell specific glucokinase |
| $GK^{L}$ | liver (hepatocyte)-specific glucokinase |
| Glc | glucose |
| GLUT | glucose transporter |
| GPI | G6P isomerase |
| GSIS | glucose-stimulated insulin secretion |
| GTT | glucose tolerance test |
| HIF-1 | hypoxia-inducible factor 1 |
| HK | hexokinase |
| HKB | yeast hexokinase B |
| HMIT | $H^{+}$-*myo*-inositol cotransporter |
| HNF | hepatocyte nuclear factor |
| Ins | insulin |
| i.p. | intra peritoneal |
| IPF1 | insulin promoter factor 1 |
| IR | insulin receptor |
| IRE | insulin response element |
| IRS | insulin receptor substrate |
| ITT | insulin tolerance test |
| kb | kilobase |
| kDa | kiloDalton |
| Km | Michaelis constant |
| MAP | mitogen-activated protein |
| MGU | muscle glucose uptake |
| MODY | maturity-onset diabetes of the young |
| mRNA | messenger RNA |
| NADPH | nicotinamide adenine dinucleotide phosphate, reduced |
| NCBI | National Center for Biotechnology Information |
| NEFA | non-esterified fatty acid (= FFA) |
| neo | neomycin phosphotransferase |
| NF-Y | nuclear factor Y |
| NMR | nuclear magnetic resonance |
| NRF-1 | nuclear respiratory factor-1 |
| nSREBP-1a | nuclear form of sterol regulatory element binding protein 1a |
| nt | nucleotide |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PEPCK | phosphoenolpyruvate carboxykinase |
| PFA | 4 % paraformaldehyde |
| PGC1 | PPARγ coactivator 1 |
| PI3K | phosphatidyl-inositol 3-kinase |
| PPAR | peroxisome proliferator-activated receptor |
| RACE | rapid amplification of cDNA ends |
| $R_g$ | tissue-specific glucose uptake index |
| RNA | ribonucleic acid |
| RT-PCR | reverse transcriptase PCR |
| SAEC | small-airway epithelial cells |
| SDS | sodium dodecylsulfate |
| SEM | standard error of the mean |
| SH2 | Src homology 2 |
| Slc2 | solute carrier family 2 (facilitated glucose transporter) |
| STZ | streptozotocin |
| SVL | superficial vastus lateralis |
| TG | triglyceride |
| TNF | tumor necrosis factor |
| Ukko | University of Kuopio knock-out |
| UTR | untranslated region |
| VDAC | voltage-dependent anion channel |
| $VO_{2max}$ | maximal oxygen consumption |

## LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to by their corresponding Roman numerals.

**I** Heikkinen S, Suppola S, Malkki M, Deeb SS, Jänne J, and Laakso M (2000) Mouse hexokinase II gene: Structure, cDNA, promoter analysis, and expression pattern. *Mamm. Genome* 11: 91-96.

**II** Heikkinen S, Pietilä M, Halmekytö M, Suppola S, Pirinen E, Deeb SS, Jänne J, and Laakso M (1999) Hexokinase II-deficient mice. Prenatal death of homozygotes without disturbances in glucose tolerance in heterozygotes. *J. Biol. Chem.* 274: 22517-22523.

**III** Fueger PT, Heikkinen S, Bracy DP, Malabanan CM, Pencek RR, Laakso M, and Wasserman DH (2003) Hexokinase II partial knockout impairs exercise-stimulated glucose uptake in oxidative muscles of mice. *Am J Physiol Endocrinol Metab.* 285: E958-E963.

**IV** Heikkinen S, Virkamäki A, Pirinen E, Peltola P, Kuulasmaa T, Jänne J, and Laakso M Glucose intolerance during exercise in partially hexokinase II deficient mice. *Submitted*.

Also, some unpublished results are presented.

**CONTENTS**

# 1 INTRODUCTION

In humans, type 2 diabetes is a common metabolic disease affecting currently more than 150 million people worldwide (Zimmet et al., 2001). It is primarily caused by β-cell dysfunction and insulin resistance (Kahn, 1994). The presence of a strong genetic component in type 2 diabetes has been demonstrated by studies in monozygotic twins and offspring of diabetic parents (Newman et al., 1987; Vauhkonen et al., 1998). In addition, the increasing incidence of type 2 diabetes is explained by obesity, lack of exercise, and dietary factors, and their interaction with genetic factors (Zimmet et al., 2001).

The molecular basis of insulin resistance has remained unknown, but recent advances in methods for generating transgenic and knock-out mice have yielded new data, particularly regarding insulin signalling. For example, the pancreatic β-cell-specific role of glucose transporter (GLUT) 2 in insulin secretion, and the role of glucokinase (GK) in both insulin secretion and a subtype of diabetes (MODY2) have been elucidated by knock-out studies (Grupe et al., 1995; Postic et al., 1999). Increasing knowledge on molecular mechanisms explaining type 2 diabetes will offer potential therapeutic targets for the prevention and treatment of this disease.

Members of the hexokinase (HK) isoenzyme family have a pivotal role in many aspects of glucose metabolism as they handle the first intracellular step in glucose utilization by phosphorylating glucose to glucose-6-phosphate (G6P) (Wilson, 1995; Wilson, 2003). While GK serves as a glucose sensor in pancreatic β-cells tightly regulating insulin secretion (Matschinsky, 1996; Postic et al., 2001), HKII participates in the insulin-stimulated glucose uptake in skeletal and cardiac muscle, and adipose tissue (Wilson, 1995; Wilson, 2003). Although glucose transport into insulin-sensitive peripheral tissues by GLUT4 is commonly viewed as the rate-limiting step in this process (Ziel et al., 1988), glucose phosphorylation by HKII can become rate-limiting in certain physiological conditions where the demand for glucose utilization is high, like during exercise (Fueger et al., 2003).

In this study, we characterized the mouse HKII gene, constructed a HKII targeting vector, and used it to generate HKII knock-out mice to investigate the role of HKII in mouse development and glucose metabolism.

# 2 REVIEW OF THE LITERATURE

## 2.1 Hexokinase II enzyme

### *2.1.1 Characteristic features*

The first intracellular metabolic step in the utilization of the main cellular fuel, glucose, is phosphorylation by a family of hexokinases (ATP:D-hexose-6-phosphotransferase, EC 2.7.1.1). By definition, these enzymes catalyze the conversion of ATP and a D-hexose to ADP and a D-hexose-6-phosphate. Hexokinases use mainly D-Glucose as substrate hexose (Appel et al., 1994), producing G6P for the downstream metabolic pathways (Fig. 1). In mammals, the family of hexokinases consists of four distinct isoenzymes, HKI – IV, of which HKIV is called GK. These enzymes, mammalian as well as others, have been studied extensively, as has been reviewed (Malkki, 1999; Postic et al., 2001; Wilson, 1995; Wilson, 2003).

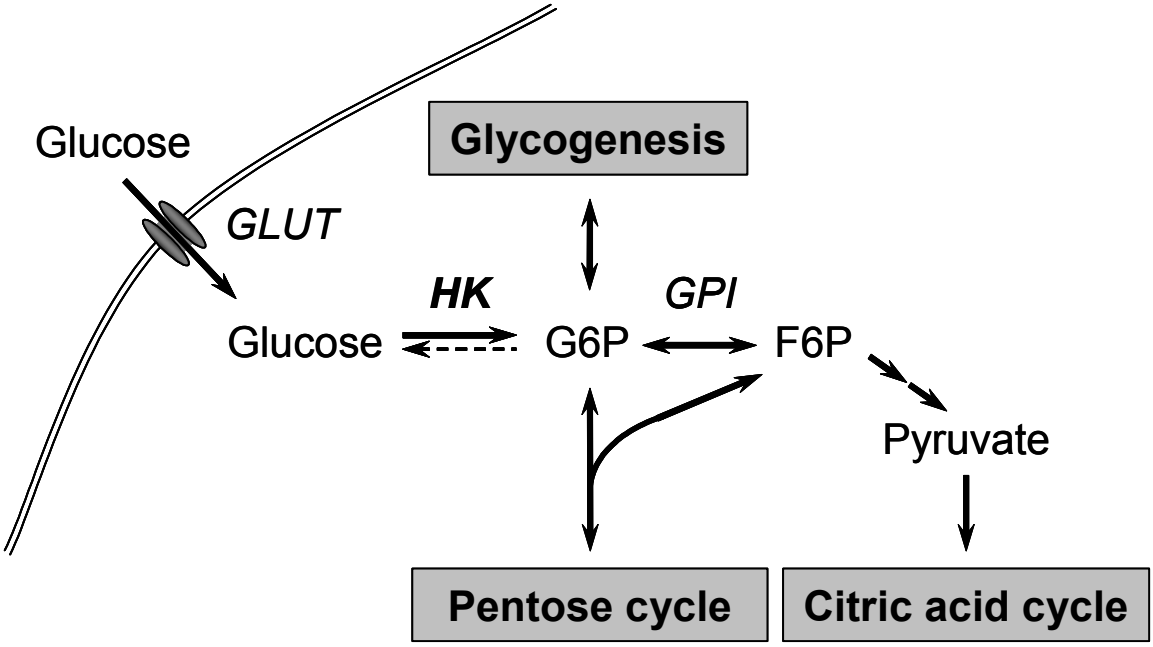



**Figure 1.** Glucose utilization pathways. Initial steps of glucose metabolism inside most cells include glucose entry, facilitated by glucose transporters (GLUT), and phosphorylation to glucose-6-phosphate (G6P) by hexokinases (HK). G6P can then be used as a substrate in the storage of energy as glycogen, in direct energy production in citric acid cycle via pyruvate, and in the synthesis of fatty acids, steroid hormones, nucleotides and nucleic acids through pentose phosphate cycle. GPI, glucose-6-phosphate isomerase; F6P, fructose-6-phosphate.

HKII, like HKI and HKIII, is 100 kDa in size, exhibits high affinity for glucose, and is subject to feedback inhibition by G6P. In rats, HKII is primarily found in skeletal and cardiac muscle and adipose tissue, *i.e.* the main sites for insulin-sensitive glucose utilization, with minor amounts present in lung and small intestine (Katzen and Schimke, 1965; Katzen et al., 1968). HKII and HKI can bind to the outer membrane of mitochondria through the interaction of hydrophobic N-

terminal sequence and porin, a voltage-dependent anion channel (VDAC) 1 (Sui and Wilson, 1997). Binding to mitochondria probably facilitates easy access to mitochondrially produced ATP (Wilson, 1995; Wilson, 2003). Alternatively, depending on the regulatory status of the cell, HKII can also be located in the cytoplasm (Wilson, 1995; Wilson, 2003). For example, in human skeletal muscle, insulin promotes a shift in HKII enzyme activity from the soluble to particulate fraction of a tissue homogenate (Vogt et al., 1998). The half-life of HKII in the tibialis anterior muscle of the rat has been determined to be 2.8 days (Weber and Pette, 1990). The characteristics of HK isoenzymes are summarized in Table 1.

**Table 1.** The characteristics of mammalian hexokinase isoenzymes.

| Isoenzyme | Molecular weight (kDA) | Gene structure | Expression | $K_i$ for G6P (mM) | $K_m$ for glucose (mM) |
|---|---|---|---|---|---|
| HKI | 100 | 18 exons | Brain, kidney, liver, heart, skeletal muscle, adipose tissue, placenta | 0.02 | ~ 0.03 |
| HKII | 100 | 18 exons | Heart, skeletal muscle, adipose tissue, lung, small intestine, many tumors | 0.02 | ~ 0.3 |
| HKIII | 100 | 18 exons | Liver, heart, lung, small intestine, leukocytes | 0.1 | ~ 0.003 |
| HKIV (Glucokinase) | 50 | 10 exons | Liver, pancreas, glucose sensing cells in brain and gut | Not inhibited | ~ 5 |

### *2.1.2 Evolution*

HKII is likely to be the origin of the other two 100 kDa HKs (HKI and HKIII). HKII itself is the end-product of an evolutionary process steming from duplication and tandem fusion of an ancestral 50 kDa gene (Kogure et al., 1993; Wilson, 2003). Indeed, there is a striking 54 % similarity between N- and C-terminal halves of the human HKII amino acid sequence (Deeb et al., 1993). Furthermore, HKII, unlike HKI and HKIII, has retained catalytic activity with susceptibility to product inhibition in both N- and C-terminal halves of the fused duplex (Wilson, 2003).

### *2.1.3 Function*

The effects of insulin on HKII expression have been studied in streptozotocin (STZ) treated rats. STZ, a nitrosourea derivative toxic to pancreatic β-cells, leads to insulin deficiency and severe hyperglycemia, similar to that in type 1 diabetes or late-stage type 2 diabetes. HKII activity is decreased in STZ-treated rat tissues, especially in white adipose tissue. The STZ-induced

decrease in HKII can be countered by insulin treatment (Burcelin et al., 1993; Katzen et al., 1970). The insulin deficiency-related decrease in HKII activity is caused by both increased degradation and decreased synthesis rates of HKII (Frank and Fromm, 1986a; Frank and Fromm, 1986b).

Regulation by the anabolic hormone insulin, together with the other characteristics of HKII enzyme, suggests that HKII serves mainly as a provider of glucose for anabolic metabolism by producing G6P for pathways leading to glycogen and lipid synthesis (Sebastian et al., 2000; Wilson, 1995; Wilson, 2003). In contrast, HKI, otherwise the most similar to HKII of the other hexokinases, is thought to provide glucose mainly for catabolic metabolism, partly because it is highly expressed in the brain which normally exhibits virtually complete dependence on glucose in highly active energy production by glycolytic metabolism (BeltrandelRio and Wilson, 1992; Wilson, 1995; Wilson, 2003). The specific biological function of HKIII, localized to the nuclear periphery as well as cytoplasm of several tissues, remains largely undefined (Preller and Wilson, 1992; Wilson, 2003). The pancreatic β-cell form of GK plays an important role in insulin secretion by sensing the level of circulating glucose, while the liver form, produced by the same gene through the use of a different promoter (Magnuson and Shelton, 1989), regulates hepatic glucose metabolism by influencing glucose utilization and glycogen synthesis, partly through the regulation of other glucose-responsive genes (Matschinsky, 1996; Postic et al., 2001).

## 2.2 Hexokinase II gene and mRNA

### *2.2.1 Structure*

The sequence and genomic structure of the rat and human HKII genes are known (Lehto et al., 1995; Malkki et al., 1994; Printz et al., 1995; Printz et al., 1993). The rat and human HKII gene has 18 exons, spans at least 55 kb, and is transcribed into a major mRNA transcript of ~5500 nt which encodes a 917-amino acid protein through an open reading frame of 2751 nt (Malkki et al., 1994; Printz et al., 1995; Printz et al., 1993; Thelen and Wilson, 1991). In addition to the 2751 nt coding sequence, the HKII mRNA in human muscle and adipose tissues contains a 431-449 nt 5’ untranslated region (UTR), a 2394 nt 3’UTR, and a poly(A) tail of approximately 200 nt (Malkki et al., 1997; Printz et al., 1995). Similarly, the 5’UTR of the rat adipocyte HKII mRNA is 462 nt and the 3’UTR 2142 nt in length (Printz et al., 1993). The similarity of coding and amino acid sequences between the rat and human HKII genes is 87 % and 94 %, respectively.

A single major transcription initiation site is present in both the rat and human HKII gene promoter (Ichihara et al., 1995; Malkki et al., 1997; Printz et al., 1993). Several minor initiation sites, up to 41 bp upstream, are also used in human and rat skeletal muscle, but not in human adipose tissue (Ichihara et al., 1995; Malkki et al., 1997). For the termination of HKII gene transcription there is a single major polyadenylation site as well as two additional sites up to ~1 kb upstream of the major site both in rat and human (Printz et al., 1995; Printz et al., 1993). The most 5' additional polyadenylation site might account for the ~4 kb minor HKII transcript sometimes seen in HKII Northern blots of normal tissue (Printz et al., 1995; Printz et al., 1993) and cancer cells (Mathupala et al., 1997b; Nakashima et al., 1988; Sebastian and Kenkare, 1998).

### *2.2.2 Chromosomal localization and pseudogenes*

By fluorescent *in situ* hybridization techniques, the rat HKII gene has been localized to chromosomal region 4q34 (Sebastian et al., 1997), and the human counterpart to 2p13.1 (Lehto et al., 1993). The mouse HKII gene has been localized to chromosome 6 by high-resolution genetic mapping (Weber et al., 1998), and more precisely, according to the information derived from the Mouse Genome project, available in the LocusLink database at the National Center for Biotechnology Information (NCBI), to chromosome 6 at region C3, or at 34.5 cM.

The human genome has a pseudogene for HKII at chromosome X (Ardehali et al., 1995). Also chromosome 4q26 has been suggested for the location of the human HKII pseudogene (Lehto et al., 1995), but the human genomic data in the NCBI databases support only the X chromosomal localization. The human HKII pseudogene lacks introns, has a high level of similarity (97%) to the open reading frame of the functional human HKII gene, and is most likely non-functional due to a premature translation stop signal at codon 120 (Ardehali et al., 1995; Malkki et al., 1994). According to currently available genomic data at the NCBI databases, neither the rat nor mouse carries pseudogenes for HKII.

### *2.2.3 Tissue distribution*

In rat, HKII mRNA is expressed in the heart, lung, skeletal muscle, and white and brown adipose tissue, but not in the kidney, brain and liver (Printz et al., 1993). In the same study, the highest HKII mRNA to HKI mRNA ratios were observed in muscle and brown adipose tissue, whereas in heart, lung and white adipose tissue, HKII and HKI were expressed approximately equally. Less is known of the human HKII mRNA tissue distribution, apart from more easily available skeletal muscle and adipose tissues where HKII mRNA is clearly present (Ducluzeau et al., 2001; Lehto et al., 1995).

Although the tissue-specificity of HKII transcription seems to be rather strict under normal circumstances, some physiological states elicit HKII mRNA expression in non-typical tissues. In many cancers, irrespective of the tissue of origin, expression of HKII is greatly (>100-fold) increased by gene amplification and promoter activation to facilitate the high glycolytic rate which is needed for the maintenance of rapid growth of these cells (Mathupala et al., 1997b; Nakashima et al., 1988; Sebastian and Kenkare, 1998). It has been recently suggested that the mitochondrial binding of large amounts of HKII, as seen in malignant cells, may promote aberrant growth by preventing the apoptotic protein Bax from inducing mitochondrial dysfunction and cell death (Pastorino et al., 2002). Detectable HKII mRNA and protein expression occurs also in mouse liver upon overexpression of the transcriptionally active nuclear form of sterol regulatory element binding protein-1a (nSREBP-1a) which has a main function as a regulator of genes participating in fatty acid and triglyceride biosynthesis (Sebastian et al., 2000). This, together with the finding that HKII mRNA is also present in lactating rat mammary gland (Kaselonis et al., 1999; Walters and McLean, 1968) underlines the anabolic role of HKII.

### *2.2.4 Regulation*

The use of alternative promoters or splicing schemes have not been shown for HKII gene in any of the studied mammalian species. In contrast, a single GK gene is expressed in different forms in pancreatic β-cells and hepatocytes through the use of alternative promoters and first exons (Magnuson and Shelton, 1989), and differential splicing produces unconventional transcripts for HKI in spermatogenic cells (Mori et al., 1998) as well as in red blood cells (Murakami et al., 1999).

The mRNA expression of HKII gene, as suggested by tissue distribution thereof, is indeed regulated by insulin. Studies in skeletal muscle (C2C12 and L6) and white and brown adipose tissue (3T3-F4422A and BFC-1B, respectively) cells *in vitro* show that insulin treatment will lead to a 2 to 6-fold increase in the HKII mRNA, due to increased transcription (shown in L6 cells) (Osawa et al., 1995; Osawa et al., 1996b; Printz et al., 1993). This effect is accompanied by a corresponding increase in HKII enzyme activity (shown in 3T3-F442A cells) (Printz et al., 1993).

Insulin regulates HKII mRNA levels through transcription also *in vivo* (Jones and Dohm, 1997). In epididymal fat pads of STZ treated rats, a 70-90 % reduction in both HKII and GLUT4 mRNA, recoverable by insulin treatment, has been demonstrated (Burcelin et al., 1993; Gerrits et al., 1993; Printz et al., 1993). However, a short-term insulin deficiency, or the countering insulin treatment, did not significantly affect HKII or GLUT4 mRNA in skeletal and cardiac

muscle (Burcelin et al., 1993). Therefore, HKII and GLUT4 mRNA are coordinately expressed in white adipose tissue where both respond readily to changes in insulin concentrations, but not to hyperglycemia. While HKII mRNA is reduced in rat tissues if insulin is lacking, the opposite is true for the excess of insulin. It has been demonstrated that a prolonged increase in circulating insulin concentration, achieved by a 24-h euglycemic-hyperinsulinemic clamp, increases HKII mRNA and enzyme activity by 2 to 8-fold in rat skeletal muscle and adipose tissue whereas GLUT4 mRNA and protein are increased by 23-fold and 2-fold, respectively, only in adipose tissue (Postic et al., 1993). A shorter, 6-h clamp resulted in similar, albeit milder effects. In both clamps, coordination of HKII and GLUT4 expression was again observed in adipose tissue, but not in skeletal muscle. HKII mRNA is increased also in human skeletal muscle already at the 120 minute time point of a 6-h clamp (2-fold) (Vogt et al., 2000), or at the end of a 4-h clamp (3-fold) (Mandarino et al., 1995). Interestingly, when hyperinsulinemia was combined with hyperglycemia in rats, no further increase in HKII mRNA was observed (Postic et al., 1993). Further evidence for the role of insulin on HKII gene expression has been provided by a study where fast-twitch muscle-specific overexpression of GLUT4 in 2 to 4-month-old mice resulted in mild insulinopenia and concomitant decrease in HKII mRNA in non-GLUT4-overexpressing tissues, *i.e.* the heart and adipose tissue, whereas in muscle the decrease was prevented, presumably due to the 2.5-fold stimulatory effect of the transgene on glucose uptake (Tsao et al., 1996a). Furthermore, insulinopenia subsided in older (7 to 9-month-old) GLUT4 transgenic mice, which lead to the normalization of HKII mRNA levels in the heart and adipose tissue.

HKII gene expression is also regulated by catecholamines through β-adrenergic receptor-mediated generation of cyclic AMP (cAMP). In L6, 3T3-F4422A and BFC-1B cells, cAMP has been shown both directly and indirectly, using the β-adrenergic agonist isoproterenol, to mediate a 2 to 4-fold increase in HKII gene expression, which is partially additive to that of insulin and is due to increased transcription (Osawa et al., 1995; Osawa et al., 1996a).

Exercise increases glucose utilization in skeletal and cardiac muscle through a variety of mechanisms, including increased blood flow, membrane glucose transport capacity (*e.g.* by signalling through 5'-AMP-activated protein kinase (AMPK) (Rutter et al., 2003)), and increased activity of glycolytic enzymes (*e.g.* HKII) (Richter et al., 2001; Wasserman and Halseth, 1998). Indeed, in rat skeletal muscle, several modes of single-bout exercise (high- and low-intensity, variable duration) commonly elicit a 3 to 5-fold increase in HKII transcription rate and mRNA level (Hildebrandt et al., 2003; O'Doherty et al., 1996; O'Doherty et al., 1994). The most striking increase in HKII transcription rate (>25-fold) was observed in red, but not in white gastrocnemius muscle immediately after the cessation of a 3 h low-intensity (~50% of maximal

oxygen consumption ($VO_{2max}$)) exercise (Hildebrandt et al., 2003). Also human skeletal muscle biopsies, analyzed before and after a 1 h moderate exercise – 3 h recovery -session, show an increase (2.1-fold) in HKII mRNA level, with comparable (1.8-fold) increase in total HKII enzyme activity (Koval et al., 1998). Furthermore, high-intensity exhaustive (~10 min) knee-extensor exercise, and short-term (1 to 1½ h) exhaustive and long-term (4 h) non-exhaustive moderate-intensity cycle exercise increase human skeletal muscle HKII mRNA and transcription rate during the first hours of recovery (up to 6-fold, compared with pre-exercise level) (Nordsborg et al., 2003; Pilegaard et al., 2000). Prior high-intensity exercise training diminishes the effect of exercise bout on the amount of HKII mRNA, presumably due to the training-increased glycogen level (Pilegaard et al., 2002), but does not affect the resting levels (Nordsborg et al., 2003). In connection with exercise, it is important to note that the HKII gene is indeed activated by AMPK signaling, because an AMP analog 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR) elicits a 2 to 4-fold increase in HKII transcription in rat gastrocnemius muscle (Stoppani et al., 2002). The mechanism by which contraction (*i.e.* exercise) stimulates HKII transcription may involve the depletion of intracellular $Ca^{2+}$ stores (Halseth et al., 2000).

Tumor growth rate often exceeds that of neovascularization, leading to hypoxic conditions within at least some parts of the tumor. The effect of hypoxia on HKII mRNA expression has been shown in A549 human lung carcinoma cells (Riddle et al., 2000) and in AS-30D cells (Mathupala et al., 2001) where the activation of HKII promoter by hypoxia was 4.1-fold, and 3-fold, respectively. Because primary cultured human small-airway epithelial cells (SAEC) also respond to hypoxia by 1.9-fold increase in HKII mRNA (Riddle et al., 2000), the regulation of HKII gene expression by hypoxia may apply also to healthy tissues in addition to tumors. Furthermore, since hypoxia also occurs in exercising muscle, and the signalling pathways for the induction of glucose uptake by hypoxia and exercise appear to be different (Wasserman and Halseth, 1998; Wojtaszewski et al., 1998), this mode of HKII regulation may be of added importance.

Factors affecting the amount of HKII mRNA, either in skeletal muscle and adipose tissue, or in tumor cells, also include glucose (Mathupala et al., 1995; Rempel et al., 1996), p53 (Mathupala et al., 1997a), glucagon (Mathupala et al., 1995), epinephrine (Jones and Dohm, 1997), the insulin-sensitizing drug pioglitazone (a member of the thiazolidinedione family) (Braithwaite et al., 1995), pregnancy (Yanase et al., 1997), and denervation (Jones et al., 1997).

### 2.2.5 Promoter characteristics

Sequence analysis of the rat and human HKII promoters have suggested the presence of several putative transcription factor binding sites within the highly conserved 450 bp proximal promoter region, including a TATA-like box at -13 bp, a CCAAT-box at -66 bp, an inverted CCAAT-box at -118 bp, and two cAMP response elements (CRE) at -51 and -286 bp relative to the human transcription initiation site (Malkki et al., 1997). The functional importance of the ~500 bp proximal promoter region for the basal promoter activity has been clearly demonstrated by transient transfection studies with different lengths of HKII promoter driving the expression of a reporter gene. In L6 myotubes, the -90 bp promoter was sufficient for full basal promoter activity (Osawa et al., 1996b). In C2C12F3 myoblasts and 3T3F422A preadipocytes, -304 and -505 bp promoters, respectively, performed similarly (Malkki et al., 1997).

Some of the intermediate players in the insulin signalling pathway leading to HKII promoter activation in skeletal muscle and adipose tissue are known, and include phosphatidyl-inositol 3-kinase (PI3K) and $p70^{S6K}$, but not the mitogen-activated protein (MAP) kinase pathway (Koval et al., 1999; Osawa et al., 1996b). The final transcription factor(s) directly activating HKII promoter, and the promoter binding site(s) thereof, are still unknown, however. Indeed, available data on the induction of HKII promoter by insulin is contradictory. In one study insulin was observed to induce the activity of the -487 bp proximal HKII promoter region by 3.5-fold in L6 myotubes, but the specific insulin response elements were not identified (Osawa et al., 1996b). In contrast, Malkki et al. demonstrated a lack of human HKII promoter induction by insulin in both C2C12F3 myoblasts and 3T3F422A preadipocytes, even with the largest reporter construct used, containing a 4 kb HKII promoter (Malkki et al., 1997). Thus the insulin response element(s) may reside outside of the 4 kb promoter segment. Furthermore, when the human 4 kb promoter construct and several shorter ones were studied *in vivo* in transgenic mice, the lack of reporter induction by insulin was verified in both skeletal muscle and adipose tissue (Pirinen et al., 2003). One reason for the contradictory results may be the different cell-lines and conditions used in the above described studies. For example, L6 cells, unlike normal skeletal muscle, are actually low in GLUT4, but high in non-insulin-sensitive GLUT1 (Osawa et al., 1995). More generally, a single universal insulin response element seems not even to exist, since promoters of a number of genes, clearly regulated by insulin at the level of transcription, contain functional insulin response elements (IRE) that are different from each other (reviewed in O'Brien et al., 2001).

A detailed analysis of the basal and cAMP mediated activation of the rat HKII promoter in L6 cells verified the importance of the previously identified putative promoter elements (Osawa et

al., 1996a). Full basal HKII promoter activity is achieved when nuclear factor-Y (NF-Y) binds to the CCAAT-box and the inverted CCAAT-box (also referred to as the Y-box), and homo- and heterodimers composed of CRE binding protein (CREB) and activating transcription factor 1 (ATF-1) bind to the CRE element closest to the TATA-box. However, the binding of NF-Y to the inverted CCAAT box is not required for the maximal cAMP-mediated promoter activation.

The functionality of the HKII promoter elements studied in L6 cells (Osawa et al., 1996a) was recently confirmed by an analysis of 4.3 kb rat HKII gene promoter in highly glycolytic rat hepatoma AS-30D cells (Lee and Pedersen, 2003). In addition to the previously confirmed binding sites, CRE modulator (CREM) was shown to bind to CRE, and transcription factors Sp1, Sp2 and Sp3 were shown to bind to four separate GC boxes within the -281 to -35 bp region relative to the rat HKII transcription start site. The Sp-family members were suggested to be of special importance for overexpression of HKII in tumor cells. It was also recently shown that in normal hepatocytes the HKII promoter is methylated at 18 CpG sites within the CpG island surrounding the transcription start site (-350 to +781 bp). In contrast, the same sites are all demethylated in AS-30D hepatoma cells, suggesting a significant role for methylation in the activation of HKII promoter at least in tumor cells (Goel et al., 2003).

In addition to the proximal region of the HKII promoter, also the distal region (approximately at -4 kb) has been shown to contain functional promoter elements. For example, the activation of HKII promoter by hypoxia has been shown to be dependent on the binding of hypoxia-inducible factor 1 (HIF-1) to two HIF-1 binding sites, overlapping putative glucose response mediating E-box sequences at -3808 and -3764 bp of the rat HKII promoter (Mathupala et al., 2001). Furthermore, in AS-30D hepatoma cells overexpressing mutant p53, a 1.5-fold activation of a cotransfected HKII promoter – reporter construct has been explained by the presence of two functional binding sites for mutant p53 at -4199 and -4253 bp relative to transcription start site of the rat HKII gene (Mathupala et al., 1997a).

## 2.3 Type 2 diabetes

### *2.3.1 Overview*

Type 2 diabetes is a common metabolic disorder currently affecting approximately 150 million people worldwide, with an estimated increase to 200 million by the year 2010 (Zimmet et al., 2001). The disorder is characterized by insulin resistance, *i.e.* the decreased ability of peripheral target tissues, especially skeletal muscle but also adipose tissue and liver, to respond properly to normal circulating concentrations of insulin, and by abnormal insulin secretion capacity of the

pancreatic β-cells, either of which may predominate (Kahn, 1994; Petersen and Shulman, 2002). Despite insulin resistance and concomitant increase in insulin secretion, euglycemia may be maintained years before the eventual fall in insulin secretion, resulting in hyperglycemia (DeFronzo et al., 1992; Kahn, 1994; Warram et al., 1990). Some insulin secretion, however, usually persists. This prevents most people with type 2 diabetes from becoming dependent on exogenous insulin, except in the later stages of the disease if normalization of blood glucose levels by life-style changes (weight loss, exercise, and diet) and oral hypoglycemic agents fails (Zimmet et al., 2001).

One of the functions of insulin is to inhibit lipolysis, *i.e.* the release of free fatty acids (FFA) from triglycerides stored primarily in white adipose tissue. Thus, insulin resistance in adipose tissue leads to increased circulating FFA levels, another characteristic feature of type 2 diabetes (Reaven et al., 1988). This potentiates hyperglycemia, since skeletal muscle prefers to oxidize FFAs rather than glucose when plasma FFA levels are high, thereby inhibiting whole body glucose utilization and oxidation (Arner, 2001; Large and Arner, 1998; Randle, 1998).

The underlying molecular defects in insulin resistance apparently reside within the network of insulin signalling pathways (Pessin and Saltiel, 2000), but alterations associated consistently with insulin resistance or type 2 diabetes remain largely unidentified. According to the current view (Saltiel and Kahn, 2001; Saltiel and Pessin, 2002; Zierath, 2002), the main message of insulin, *i.e.* the uptake of glucose into skeletal muscle and adipose tissue and the attenuation of glucose production in liver, is intracellularly communicated to appropriate metabolic machinery through a complex network of insulin signalling pathways (Fig. 2). The binding of insulin to insulin receptor at the outer cell membrane is followed by receptor autophosphorylation. Insulin receptor is then able to phosphorylate several other signalling molecules, like the insulin receptor substrate (IRS) proteins, which recruit further signalling proteins containing Src homology 2 (SH2) domains (Saltiel and Pessin, 2002). From there, insulin signal is carried on by numerous downstream mediators and modulators, of which many are known, to final destinations, including general regulation of gene expression (MAP pathway), cell growth and differentiation (MAP and phosphatidyl-inositol 3-kinase (PI3K) pathways), metabolism of glucose, lipids, and protein, and the expression of specific genes (PI3K pathway). In addition, glucose transport is increased in response to increased demand for glucose uptake via insulin stimulated translocation of GLUT4 from storage vesicles to the cell membrane (PI3K/Akt and Cbl/CAP pathways) (Bryant et al., 2002; Jhun et al., 1992; Saltiel and Pessin, 2002).

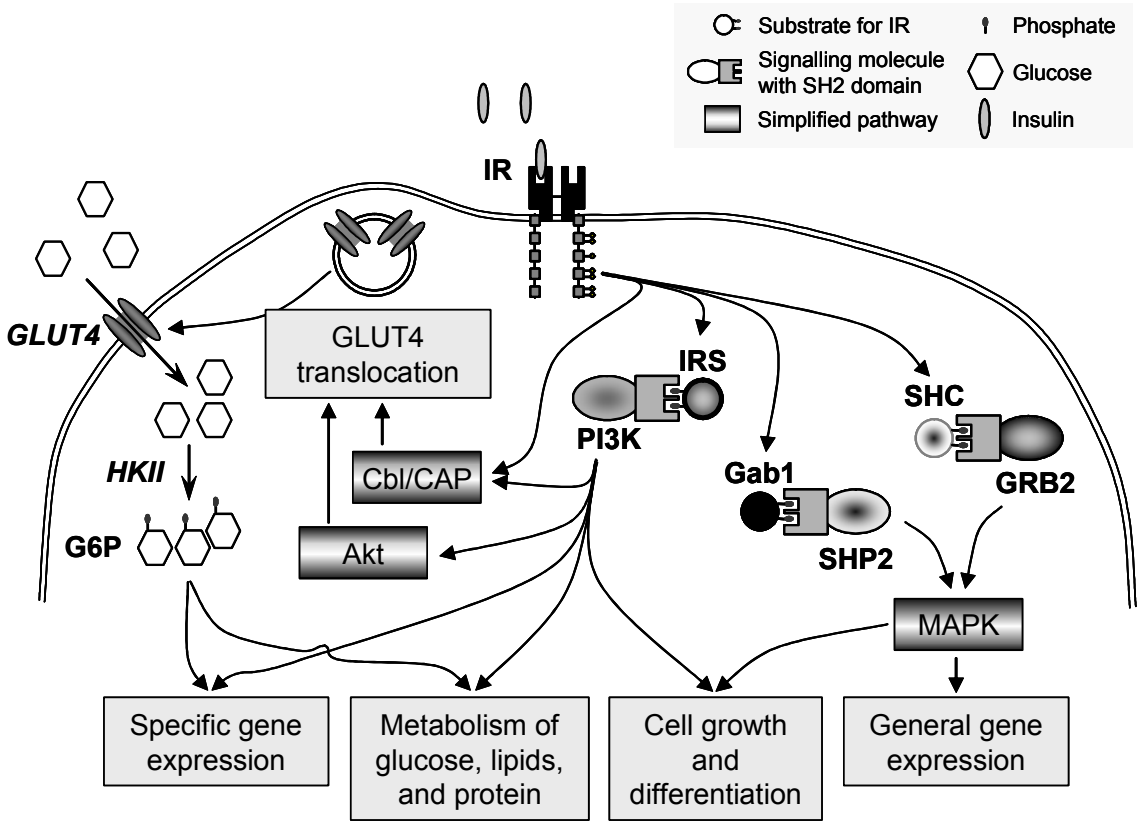



**Figure 2.** Overview of insulin signalling in skeletal muscle.

Recently, the first consistent molecular alteration in type 2 diabetes was found when the mRNA expression of nuclear respiratory factor-1 (NRF-1) and peroxisome proliferator-activated receptor (PPAR) γ coactivator 1 (PGC1), participating in the regulation of several key enzymes in oxidative metabolism and mitochondrial function, was shown to be mildly reduced in skeletal muscle in subjects with type 2 diabetes by microarray and quantitative PCR techniques (Mootha et al., 2003; Patti et al., 2003). Most importantly, PGC1 expression was consistently reduced also in non-diabetic subjects with family history of diabetes (Patti et al., 2003). The observed pattern of NRF-1 and PGC1 expression was suggested to be a primary characteristic in the prediabetic state. Thus, reduced expression of PGC1, in addition to clinically measurable aspects of respiratory capacity, might be used as a marker for prediabetic condition. In addition, the underlying molecular events may provide pharmacological targets for prevention and treatment of type 2 diabetes.

### *2.3.2 Genetic studies*

There is a strong genetic component in type 2 diabetes, as shown in studies in monozygotic twins and offspring of diabetic parents (Haffner et al., 1988; Newman et al., 1987; Vauhkonen et al., 1998). Identifying major genes for type 2 diabetes has proven to be difficult. In most cases it

has been difficult to confirm the original associations observed between type 2 diabetes or insulin resistance and variants of candidate genes for malfunctioning insulin action or insulin secretion (Elbein, 2002). One interesting case does exist, the relatively common proline to alanine substitution at codon 12 (P12A) (alanine allele frequency ~15 %) of the PPAR$\gamma_2$ isoform. While altering gene function and insulin sensitivity by reducing the ability to bind to and transactivate the PPARγ response element, PPAR$\gamma_2$ P12A variant paradoxically decreases the risk of type 2 diabetes and associates with lower body mass index (BMI) (Altshuler et al., 2000; Deeb et al., 1998).

As a part of the candidate gene approach, HKII has also been studied. Naturally occurring variants of the human HKII gene promoter or protein coding region do not contribute to insulin resistance (Echwald et al., 1995; Laakso et al., 1995; Lehto et al., 1995; Malkki et al., 1997; Malkki et al., 1998; Taylor et al., 1996; Vidal-Puig et al., 1995), nor is there any evidence for the involvement of HKII deficiency in any other disorder. Also HKIII has not been linked with any disorder. In contrast, certain rare sequence variants of the human HKI gene cause nonspherocytic hemolytic anemia (Bianchi et al., 1997; Magnani et al., 1986), and variants of the GK gene cause a rare subtype of type 2 diabetes (Fajans et al., 2001; Froguel et al., 1993).

Genome-wide scans have had limited success in identifying risk genes for type 2 diabetes. In a multitude of studies, reviewed in (Elbein, 2002), only one type 2 diabetes susceptibility gene, calpain-10, has been identified after the initial discovery (in Mexican-Americans) of the susceptible chromosomal region (Hanis et al., 1996; Horikawa et al., 2000), but even that seems to be lacking association with type 2 diabetes in some other populations, for example in Japanese (Horikawa et al., 2003) and in Scandinavian Caucasians (Rasmussen et al., 2002).

Identification of potential susceptibility loci and candidate genes may prove to be important, since different variants may have synergistic effects. For example, synergy between the loci on chromosomes 2q (calpain-10) and 15 (near Cyp19 gene) leads to increased diabetes susceptibility in Mexican Americans (Cox et al., 1999). Similarly, mice with heterozygous mutations in both insulin receptor (IR) and insulin receptor substrate (IRS) 1 genes (IR/IRS($^{+/-}$) mice) develop diabetes more often than mice with heterozygous mutations in either gene alone (Bruning et al., 1998).

Several rare monogenic subtypes of type 2 diabetes have been identified. Autosomal dominant forms of type 2 diabetes, called MODY (maturity onset diabetes of the young) (Fajans et al., 2001; Froguel et al., 1993), result from mutations in genes for GK (Froguel et al., 1993), hepatocyte nuclear factor (HNF) 1α, 4α, and 1β (Horikawa et al., 1997; Yamagata et al., 1996a;

Yamagata et al., 1996b), insulin promoter factor 1 (IPF1) (Stoffers et al., 1997), and NeuroD1 (Malecki et al., 1999). Additional MODY genes are likely, since the above mentioned explain, on average, less than 80 % of all cases of MODY. Another genetically defined form of type 2 diabetes results from mutations in mitochondrial DNA. One example of these is the mutation in the leucine transfer RNA at position 3243 (Kadowaki et al., 1994). Although the monogenic subtypes account only for less than 5 % of all type 2 diabetes cases, they can provide important insights to the molecular mechanisms underlying diabetes.

Interestingly, all identified MODY genes save GK are transcription factors participating in the regulation of gene expression. So is the previously mentioned PPAR$\gamma_2$, a variant of which is associated with decreased risk for type 2 diabetes, and NRF-1 and PGC1, expression of which is consistently reduced in type 2 diabetes. This may suggest that it might be beneficial to prefer transcription factor genes when selecting candidates for mutation screening.

### *2.3.3 Hexokinase II in type 2 diabetes*

Insulin-induced glucose uptake (Baron et al., 1991; Pendergrass et al., 1998), the rates of glucose oxidation and non-oxidation (Del Prato et al., 1993; Kruszynska et al., 1998), and intracellular G6P levels (Rothman et al., 1992) are all reduced in type 2 diabetes, particularly in skeletal muscle, which has a major role in whole-body glucose disposal (DeFronzo et al., 1992). In addition to abnormal glycogen synthesis in skeletal muscles of subjects with type 2 diabetes (Shulman et al., 1990; Thorburn et al., 1991), simultaneous defects either in glucose transport by GLUT4, commonly viewed as the rate-limiting step in basal glucose utilization, or in glucose phosphorylation by HKII, may also contribute to reduced glucose uptake (Bonadonna et al., 1996; Chang et al., 1996; Fink et al., 1992; Halseth et al., 1998; Halseth et al., 1999; Hansen et al., 1995; Hansen et al., 2000, Rossetti, 1990; Katz et al., 1991; Kubo and Foley, 1986; Ren et al., 1993; Saccomani et al., 1996; Ziel et al., 1988).

Insulin activates HKII mRNA transcription, protein synthesis and enzyme activity in healthy rats, mice and humans (Postic et al., 1993; Tsao et al., 1996a; Vogt et al., 2000). However, in an experimental model for type 2 diabetes, the KKA$^Y$–mouse (Wehner et al., 1972), HKII mRNA and protein are reduced by 34 to 71 % in adipose tissue and skeletal muscle, with concordant reduction in GLUT4 in adipose tissue, but not in skeletal muscle (Braithwaite et al., 1995). Since treatment with pioglitazone, an insulin-sensitizing thiazolidinedione drug, was able to ameliorate the effects of diabetes on HKII and GLUT4 mRNA and protein, abnormal expression of HKII is likely to occur secondary to the diabetic state (Braithwaite et al., 1995). Accordingly, in skeletal muscles of type 2 diabetic subjects, reduced levels of HKII enzyme activity, protein, and mRNA

(Kruszynska et al., 1998; Pendergrass et al., 1998) are more or less unresponsive to euglycemic hyperinsulinemia (Kruszynska et al., 1998; Pendergrass et al., 1998; Vestergaard et al., 1995). It is possible that in insulin resistant muscle, the feedback inhibition of HKII by G6P is not reduced by insulin like in normal, insulin-sensitive muscle, as shown in isolated soleus muscles of obese (insulin-resistant) and lean (insulin-sensitive) Zucker rats (Sanderson et al., 1996).

In skeletal muscle of both type 2 diabetic and non-diabetic obese subjects, compared with lean controls, exercise elicits a 1.7 to 1.9-fold increase in HKII mRNA, but not in HKII enzyme activity, suggesting the presence of a posttranscriptional defect in the regulation of HKII by exercise in type 2 diabetes as well as in obesity (Cusi et al., 2001; Koval et al., 1998).

## 2.4 Genetically altered glucose transport and phosphorylation in mice

Animal models over- or underexpressing genes of interest have facilitated major advances in the study of genetic factors underlying type 2 diabetes. Especially studies in knock-out mice have elucidated the roles of various genes involved in insulin signalling, transcriptional regulation of gene function, and hormonal communication between tissues. Further advances have been made by targeting gene modifications to specific tissues or cell types, and by combining different gene modifications into a single mouse model (e.g. double or even triple knock-out mice). Although the majority of the genetically modified rodent models of type 2 diabetes do not display all of the characteristics of the disease, the increase in the understanding of the underlying molecular mechanisms of type 2 diabetes has been considerable, inasmuch as the results from mice are applicable to humans.

Glucose transport over the plasma membrane occurs to an extent by diffusion, but is mainly controlled by a 13-strong family of bidirectional glucose and polyol transporter molecules, including GLUT1 to GLUT12 and $H^+$-*myo*-inositol cotransporter (HMIT), and recently classified as 'solute carrier family 2 (facilitated glucose transporter)' (Slc2) (Uldry and Thorens, 2003). GLUT1 (Slc2a1) is expressed in most cell types and has affinity for glucose within the lower physiological range (Km ~3 mM) (Uldry and Thorens, 2003). In muscle and heart, GLUT1 appears to primarily mediate the basal glucose transport (Mueckler, 1990). GLUT2 (Slc2a2) is expressed in pancreatic β-cells, hepatocytes, and the absorptive epithelial cells of the kidney and intestine, and has a low affinity for glucose ($K_m$ ~17 mM) (Uldry and Thorens, 2003). GLUT2 has been thought to play an important role in the control of glucose-stimulated insulin secretion (GSIS) (Unger, 1991). GLUT4 (Slc2a4) is expressed in skeletal muscle, heart, and adipose tissue, and has affinity for glucose well within the physiological range ($K_m$ ~5 mM)

(Uldry and Thorens, 2003). Insulin and exercise-stimulated glucose transport are the main functions of GLUT4 (Bryant et al., 2002) and it partners with HKII *e.g.* in tissue distribution and insulin responsiveness (Postic et al., 1994; Wilson, 1995). Subsequent to transport into the cell, glucose is rapidly phosphorylated to G6P by the members of the hexokinase isoenzyme family (HKI-III and GK) having different tissue distributions and metabolic roles (Postic et al., 2001; Wilson, 2003). The altered expression of GLUT1, GLUT2, GLUT4, yeast hexokinase B (HKB), GK, and HKII has been extensively studied in genetically modified mice almost exclusively in relation to aspects of diabetes. The effects of genetically altered glucose transport and phosphorylation on mouse phenotype, reviewed in detail below, are summarized at the end of the literature review (Table 2).

### *2.4.1 Overexpression of glucose transporters*

Mice overexpressing GLUT1 specifically in skeletal muscle show striking increase in basal muscle glucose uptake and muscle glycogen, glucose, and lactate content, but unaltered muscle G6P content (Gulve et al., 1994; Marshall et al., 1993; Ren et al., 1993). This suggests that significant levels of both glycogen synthesis and glycolysis occur, but glucose phosphorylation is rate-limiting in these mice. GLUT1 overexpression results also in systemic effects like fasting and fed hypoglycemia and markedly improved glucose tolerance, but does not alter circulating levels of insulin or glucagon (Gulve et al., 1994; Marshall et al., 1993; Ren et al., 1993). Whole body glucose utilization is also enhanced, and the development of hyperglycemia and glucose intolerance with a high-fat, high-sugar diet is prevented (Marshall et al., 1999). Remarkably, insulin partially fails to increase glucose uptake in isolated GLUT1 transgenic muscles (Etgen et al., 1999; Gulve et al., 1994; Hansen et al., 1998; Marshall et al., 1993), despite normal cell-surface content of GLUT4 (Etgen et al., 1999; Hansen et al., 1998). This suggests that the catalytic activity of GLUT4 is decreased in GLUT1-overexpressing muscles due to a novel, as yet unknown regulatory mechanism (Hansen et al., 1998).

Cardiac-specific GLUT1 overexpression increases cardiac rates of basal glucose uptake and glycolysis which, instead of compromising cardiac function, protects experimentally hypertrophied hearts against fatal contractile dysfunction and left ventricular dilation (Liao et al., 2002).

Overexpression (~10-fold mRNA, 2 to 4-fold protein) of GLUT4 in mice (Olson et al., 1993) clearly demonstrates the importance of this transport molecule in glucose homeostasis. GLUT4 mice demonstrate fasting hypoglycemia (50 to 75 % of controls), parallel hypoinsulinemia, a striking improvement in oral glucose tolerance, and a marked increase in muscle glycogen

content and plasma levels of lactate, triglycerides, β-hydroxybutyrate and FFAs (Deems et al., 1994; Hansen et al., 1995; Liu et al., 1993; Marshall and Mueckler, 1994; Treadway et al., 1994). In GLUT4 mice, whole body glucose clearance, glucose transport rate of muscle and adipose tissue, and glucose infusion rate during the euglycemic-hyperinsulinemic clamp are increased, but, despite fasting hypoglycemia, hepatic glucose output is normal (Deems et al., 1994; Hansen et al., 1995; Marshall and Mueckler, 1994; Treadway et al., 1994). Hypoglycemia and hypoinsulinemia result in compensatory lipolysis and muscle glycogenolysis during fasting (Treadway et al., 1994). Not only insulin, but also contraction stimulates glucose transport into GLUT4 overexpressing muscle (Hansen et al., 1995). In GLUT4 mice, glucose flux through the hexosamine pathway is not increased, unlike in GLUT1 mice in which it may contribute to insulin resistance (Buse et al., 1996). Upon high-fat, high-sugar diet GLUT4 mice become obese but are protected from fasting hyperglycemia, glucose intolerance, and partially also from obesity-induced insulin resistance (Marshall et al., 1999).

A minimal, ~10 % overexpression of myc-tagged functional GLUT4 (GLUT4myc) in mice does not alter glucose metabolism, but allows easy detection of the myc-tagged GLUT4 on the cell surface (Konrad et al., 2002). Using isolated brown adipocytes from GLUT4myc mice, it has been shown that insulin-stimulated glucose uptake surpasses GLUT4 translocation by 3-fold, suggesting that insulin not only regulates GLUT4 translocation, but also somehow increases the activity of GLUT4 (Konrad et al., 2002).

In order to distinguish the role of altered muscle glucose transport efficiency from that of adipose tissue, mice overexpressing GLUT4 in skeletal muscle have been generated (Tsao et al., 1996b). They demonstrate that the majority of phenotypic effects seen in the “whole-body” GLUT4 transgenic mice is specifically due to muscle GLUT4 overexpression. For example, muscle-GLUT4 mice have improved glucose tolerance, whole-body glucose disposal, and insulin-stimulated muscle glucose uptake, and they are hypoglycemic and hypoinsulinemic (Leturque et al., 1996; Tsao et al., 1996a; Tsao et al., 1996b). The changes in glucose and insulin appear to be age-dependent (Tsao et al., 1996a; Tsao et al., 1996b). Lipid metabolism of muscle-GLUT4 mice is also affected (Tsao et al., 1996b). GLUT4-overexpressing fast-twitch muscle somewhat adapts to an increase in glucose influx by increasing muscle glycogen content as well as the rate of glycolysis (males only) or glycogen synthesis (females only). Muscle FFA oxidation is also reduced, leading to increased liver content of fat (Tsao et al., 2001). Interestingly, in conditions encouraging physical activity, muscle-GLUT4 mice exercise voluntarily four times as much as control mice while efficiently compensating the increased energy consumption by increasing food intake (Tsao et al., 2001).

Although muscle-specific overexpression of GLUT4 explains most of the phenotypic features seen in "whole-body" GLUT4 transgenic mice, also constitutive adipose tissue-specific GLUT4 overexpression results in enhanced glucose tolerance and fasting hypoglycemia (Shepherd et al., 1993). This is due to massively increased basal and insulin-stimulated glucose transport in transgenic adipose tissue which leads to increased body fat content. Increased adiposity occurs solely via adipocyte hyperplasia (>2-fold) and completely without hypertrophy, which is unexpected, since obesity is a result of increased fat cell size, and increased fat cell number is seen only in addition to hypertrophy in severe obesity (Shepherd et al., 1993). Adipocyte-GLUT4 mice are protected from further adiposity and increased body fat content induced by high-fat diet, but not from impaired glucose tolerance, most likely due to obesity-induced insulin resistance in muscle and liver (Gnudi et al., 1995). However, upon STZ-induced insulinopenic diabetes, adipocyte-GLUT4 mice have improved insulin action, probably through continued presence of high GLUT4 protein levels and glucose uptake in adipocytes (Tozzo et al., 1997).

### *2.4.2 Deficiency of glucose transporters*

Complete deficiency of GLUT2 leads to early diabetes and death within three postnatal weeks, whereas heterozygous GLUT2-deficient mice are indistinguishable from wild-type mice (Guillam et al., 1997). The phenotype of still-surviving, albeit growth-retarded, 2-week-old GLUT2-null mice resembles human type 2 diabetes since the mice are hyperglycemic, have relative hypoinsulinemia, and marked elevation in circulating FFA levels. In addition, they have severe glucosuria, presumably due to a lack of GLUT2 in the kidney, and markedly elevated circulating glucagon and β-hydroxybutyrate levels. GLUT2-null β-cells *in vitro* have reduced glucose utilization and a complete lack of first-phase, and partially also of second-phase, GSIS (Guillam et al., 1997). The defect in insulin secretion is GLUT2-specific, since reexpression of GLUT2 in β-cells completely restores GSIS (Guillam et al., 2000; Guillam et al., 1997). Thus, GLUT2 is required for GSIS, and GSIS is required for postnatal survival (Thorens, 2003).

Permanent rescue of GLUT2-null pups is achieved by expressing GLUT1 or GLUT2 transgenes selectively in β-cells of GLUT2-null mice (Thorens et al., 2000). Indeed, this restores normal growth and survival, glycemia in the fed state, and glucose tolerance. It also normalizes both *in vivo* and *in vitro* insulin secretion as well as β-cell glucose utilization. However, rescued GLUT2-null mice still have hypoinsulinemia and hyperglucagonemia in the fed state. Also severe glucosuria persists, and may contribute to the fasting hypoglycemia of rescued mice (Thorens et al., 2000). Livers of rescued GLUT2-null mice are hyperplasic and have impaired mobilization of glycogen stores upon fasting, but have normal glucose output, suggesting a

presence of a transporter-independent mechanism for glucose release in the liver, perhaps based on membrane trafficking (Burcelin et al., 2000; Guillam et al., 1998). Since the restoration of normal β-cell insulin secretion and glucose utilization is achieved by both GLUT1 and GLUT2, regardless of their different Km for glucose, GSIS depends more on the absolute glucose flux than the transporter affinity (Thorens, 2003; Thorens et al., 2000). Rescued GLUT2-null mice have also been useful in elaborating the nature of extrapancreatic glucose sensing units in the liver (Hevener et al., 1997) and in the central nervous system (Burcelin and Thorens, 2001; Thorens, 2003).

Heterozygous "whole-body" GLUT4-deficiency ($GLUT4^{+/-}$) results in peripheral, but not hepatic insulin resistance and diabetes in male mice when GLUT4 expression begins to decline from wild-type levels at 2 months of age (Rossetti et al., 1997b; Stenbit et al., 1997). Upon aging, an increasing percentage of male $GLUT4^{+/-}$ mice become hyperglycemic and hyperinsulinemic (Stenbit et al., 1997), leading to decreased muscle glucose uptake, hypertension, cardiac hypertrophy, and liver steatosis. In addition, $GLUT4^{+/-}$ adipocyte size is increased, but epididymal fat pad and body weights are normal. Thus, male $GLUT4^{+/-}$ mice are a good model to study the development of type 2 diabetes free of obesity-related complications (Stenbit et al., 1997). Furthermore, as analyzed by a euglycemic-hyperinsulinemic clamp, $GLUT4^{+/-}$ mice are clearly insulin resistant, but hepatic glucose fluxing and output are normal (Rossetti et al., 1997b).

It is interesting to note that the diabetic phenotype of $GLUT4^{+/-}$ mice largely resembles that of the polygenic mouse model for type 2 diabetes, IR/IRS($^{+/-}$) mouse (Bruning et al., 1998; Stenbit et al., 1997). Similar diabetes results also from altered levels of proteins like syntaxin 4 (heterozygous knock-out) (Yang et al., 2001) and Munc18c (overexpression) (Spurlin et al., 2003) which both take part in membrane trafficking regulating the amount of GLUT4 at the plasma membrane.

Complete deficiency of GLUT4 in GLUT4-null mice does not lead to diabetes, but does result in abnormal glucose and lipid metabolism, severe morphological abnormalities, and decreased longevity (Katz et al., 1995; Ryder et al., 1999a; Ryder et al., 1999b; Stenbit et al., 1996; Stenbit et al., 2000). In male GLUT4-null mice, the basal glucose uptake and glycogen synthesis in soleus muscle are increased but insensitive to stimulation by insulin (Stenbit et al., 1996). Male GLUT4-null mice also have increased insulin and decreased lactate and FFA levels in the fed state, and decreased level of β-hydroxybutyrate in the fasted state (Katz et al., 1995). The oral glucose tolerance of GLUT4-null mice is more or less normal while insulin sensitivity is reduced

(Katz et al., 1995). Morphological abnormalities of GLUT4-null mice include growth-retardation, severe cardiac hypertrophy (a likely contributor to the decreased longevity), and dramatically reduced adipose tissue size, which contrasts the effects of GLUT4 overexpression in adipose tissue (Shepherd et al., 1993), and offers a possible explanation for why the lipid profile of GLUT4-null mice is opposite to that which is common in type 2 diabetes (DeFronzo and Ferrannini, 1991; Katz et al., 1995).

In contrast to males, glucose uptake in the soleus muscles of female GLUT4-null mice remains insulin-sensitive despite complete loss of GLUT4 (Stenbit et al., 1996). These muscles have thus been used to show that the mechanism for the alternative insulin-sensitive glucose transport is likely to be a glucose transporter, but that the suspect transporter it is not GLUT1, 3, or 5 (Ryder et al., 1999a). For hypoxia, thought to mimic the effects of contraction in muscle glucose uptake, compensation in GLUT4-null muscles does not exist (Zierath et al., 1998). Surprisingly, partial compensation of a yet unknown nature (not GLUT1) does exist for actual exercise-stimulated glucose uptake, although the replenishment of glycogen stores after exercise is delayed (Ryder et al., 1999b).

Both GLUT4$^{+/-}$ and GLUT4-null mice can be very effectively rescued from diabetes by fast-twitch muscle-specific overexpression of GLUT4, with the exception of disturbances in adipose tissue (Tsao et al., 1997; Zierath et al., 1998). This highlights the potential importance of preserving proper levels of muscle GLUT4 expression in prevention of diabetes.

An alternative to tissue-specific transgenic rescue of "whole-body" knock-outs is the tissue-specific inactivation of the target gene using Cre-lox technology (Lakso et al., 1996; Sauer and Henderson, 1988). Muscle-specific GLUT4 knock-out mice have an age-associated diabetic phenotype similar to that of GLUT4$^{+/-}$ mice, although their diabetes is even more severe. For example, they have insulin resistance also in the liver. The reason for this phenotypic difference is currently unknown (Burcelin et al., 2003; Kim et al., 2001; Zisman et al., 2000). Heart-specific GLUT4 knock-out mice are normal except for certain cardiac pathologies like the lack of insulin-stimulated glucose uptake, and mild hypertrophy without fibrosis. The mildness of their phenotype strongly suggests that reduced substrate supply and hyperinsulinemia contribute to the cardiac phenotype of GLUT4-null mice (Abel et al., 1999). Adipose tissue-specific GLUT4 knock-out mice have reduced insulin-stimulated glucose uptake in adipose tissue, and insulin resistance in the liver and skeletal muscle. The mechanism for the liver and muscle phenotype is unknown, but may include chronic hyperinsulinemia or changes in the release of an as yet unidentified adipose-derived factor (Abel et al., 2001).

### 2.4.3 Yeast hexokinase B as a transgene

The first transgenic model with any hexokinase gene was one where the high-affinity yeast hexokinase B (Stachelek et al., 1986) was expressed in mouse pancreatic β-cells under the rat insulin II promoter (Epstein et al., 1992). These mice were generated to test the hypothesis that GK, the natural, low affinity HK of β-cells, acts as a β-cell glucose sensor in the control of insulin secretion. This, and subsequent *in vitro* studies with islets obtained from these mice (Voss-McCowan et al., 1994; Xu and Epstein, 1996) contributed to establishing that GK indeed sets the rate of insulin secretion as well as determines the level of circulating glucose at which insulin secretion begins to increase. Yeast hexokinase B has also been expressed in the mouse heart, where it increases glycolysis and glycogen storage (Liang et al., 2002). This supports the contention that phosphorylation can become rate-limiting in glucose utilization, at least in the mouse heart.

### 2.4.4 Overexpression of glucokinase

GK, like the other hexokinases, phosphorylates glucose to G6P. GK has an affinity for glucose within the physiological range, is half the size (~50 kDa) of the other hexokinases, and lacks sensitivity to inhibition by G6P (Postic et al., 2001). The GK gene is expressed through two distinct promoters, of which the upstream promoter governs expression in pancreatic β-cells and certain cells in the brain and gut while the downstream promoter directs GK expression into the liver (Jetton et al., 1994; Magnuson and Shelton, 1989; Postic et al., 2001). The pancreatic β-cell form of GK plays an important role in insulin secretion by sensing the level of circulating glucose, while the liver form regulates hepatic glucose metabolism by influencing glucose utilization and glycogen synthesis (Matschinsky, 1996; Postic et al., 2001).

An 83 kb fragment of mouse genomic DNA has been used as a transgene to overexpress GK according to the natural expression pattern in both pancreatic β-cells and liver (Niswender et al., 1997a; Niswender et al., 1997b). Under basal conditions, GK mice have mild, transgene copy number-dependent decrease in the fasting plasma glucose concentration (one extra copy: -25 %, two extra copies: -37 %), and their glucose tolerance is enhanced in parallel. With one extra copy of GK, these mice have elevated glucose clearance rate (by 21 %), but unaltered plasma insulin levels. During the hyperglycemic clamp, glucose clearance is normalized, but the plasma insulin level is decreased by ~50 %, compared with controls, and net glycogen synthesis in the liver is almost quadrupled while in muscle it is decreased by 40 %. These data show that GK overexpression leads to increased hepatic glucose metabolism, but does not increase insulin secretion because of hypoglycemia-reduced down-regulation of the islet GK content (Niswender

et al., 1997b). On a high-fat diet, mice overexpressing GK become as obese as controls, but are protected from obesity-induced type 2 diabetes due to prevention of hyperglycemia and hyperinsulinemia (Shiota et al., 2001).

The effects of liver-specific overexpression of GK have been investigated using either conventional transgene approach in mice (Ferre et al., 1996a; Ferre et al., 1996b; Hariharan et al., 1997; Jackerott et al., 2002) or adenovirus-mediated gene transfer in rats (O'Doherty et al., 1999). The promoter of phosphoenolpyruvate carboxykinase (PEPCK) has been used to increase GK expression in the livers of starved or STZ-diabetic mice (Ferre et al., 1996a; Ferre et al., 1996b). In starved PEPCK/GK mice, GK activity was 4-fold of that in control mice, and hepatic glycogen accumulation and lactate content were increased. In addition, glucose and insulin levels were decreased, and glucose tolerance was improved (Ferre et al., 1996b), and the effects of STZ-diabetes were corrected due to an increase in glycolysis while gluconeogenesis and ketogenesis were blocked (Ferre et al., 1996a). Also a modest, 20 % increase in hepatic GK activity, resulting from apolipoprotein A-I gene enhancer –directed GK transgene expression, results in similar, albeit milder modifications in the liver as well as in systemic glucose metabolism (Hariharan et al., 1997). Further similarities are seen in rats overexpressing GK via adenovirus-mediated gene transfer as these AdCMV-GKL-rats are hypoglycemic and hypoinsulinemic, apparently in AdCMV-GKL dose-dependent manner, and have increased circulating triglyceride and FFA levels, and increased fasted liver glycogen content (O'Doherty et al., 1999). Overexpression of GK in the liver, through the phenylalanine hydroxylase promoter, improves the extremely diabetic phenotype of IR-null mice (Joshi et al., 1996), but does not delay the development of ketoacidosis and subsequent early postnatal death of these mice (Jackerott et al., 2002).

Recently GK has been overexpressed by gene transfer techniques in mouse muscle or adipose tissue either alone or in combination with insulin gene (Munoz et al., 2003; Otaegui et al., 2000; Otaegui et al., 2003). Overexpression of GK in skeletal muscle, with or without concomitant expression of insulin, protects from most of the adverse effects of STZ-induced diabetes (Otaegui et al., 2000) as well as those from high-fat diet (Otaegui et al., 2003). Also adipose-specific GK overexpression with concomitant expression of insulin may counteract hyperglycemia (Munoz et al., 2003). Adenovirus-mediated GK gene transfer into rat skeletal muscle also enhances insulin-sensitive glucose uptake and whole body glucose tolerance (Jimenez-Chillaron et al., 1999; Jimenez-Chillaron et al., 2002), but unfortunately it is not capable in preventing the development of diabetes, at least when it is associated with severe obesity in diabetic Zucker fatty rats (Jimenez-Chillaron et al., 2002).

### 2.4.5 Deficiency of glucokinase

Consistent with the phenotype of patients who have MODY2 due to heterozygous GK mutations (Fajans et al., 2001), mice with heterozygous "whole-body" knock-out of the GK gene show only mildly elevated fasting glucose levels, decreased glucose tolerance, decreased relative *in vivo* insulin secretion, and abnormal liver glucose metabolism (Bali et al., 1995; Grupe et al., 1995; Postic et al., 1999). The phenotype of $GK^{+/-}$ mice is more pronounced than that of RIP-GKRZ mice, which have normal GK expression in the liver, but a 70 % reduction in pancreatic β-cell GK expression due to β-cell-specific GK ribozyme expression (Efrat et al., 1994; Rossetti et al., 1997a). For example, RIP-GKRZ mice have normal suppression of hepatic glucose production during hyperglycemia, while in $GK^{+/-}$ mice the suppression is impaired (Rossetti et al., 1997a). On a chronic high-fat diet, $GK^{+/-}$ mice become as obese and insulin resistant as high-fat fed wild-type mice. They develop severe diabetes, however, due to a lack of compensatory hyperinsulinemia, which results from the inability to increase β-cell mass by hyperplasia (Terauchi et al., 2003). In contrast, although $GK^{+/-}$ islets have impaired insulin secretion also *in vitro*, they appear to be protected from glucose toxicity, possibly providing clues to why the mild hyperglycemia in MODY2 patients does not get worse over time (Sreenan et al., 1998). A detailed study of GK-deficient β-cells has revealed that the defective insulin secretion may arise from the defective regulation of ATP-sensitive potassium channels (Sakura et al., 1998). Taken together, the results from $GK^{+/-}$ mice show that both hepatic and β-cell forms of GK have a key role in the control of glucose homeostasis, and implicate both the liver and pancreatic β-cells in MODY2 (Bali et al., 1995; Postic et al., 1999).

In humans, a complete GK-deficiency due to a homozygous GK mutation results in fetal growth retardation and massive hyperglycemia at birth, but is treatable by insulin therapy (Njolstad et al., 2001). Complete GK-deficiency in mice results in early postnatal death from severe diabetes which responds poorly to insulin therapy (Grupe et al., 1995; Postic et al., 1999). The phenotypic features of $GK^{-/-}$ pups include severe hyperglycemia within a day of birth (~8-fold), ~70 % reduction in plasma insulin levels, markedly elevated circulating triglyceride, FFA, and cholesterol levels, and microvesicular steatosis and reduced glycogens stores of the liver. Interestingly, the simultaneous inactivation of the two mouse insulin genes results in a phenotype nearly identical to that of $GK^{-/-}$ pups (Duvillie et al., 1997). The pups of one independent GK knock-out mouse line die already at mid-gestation (Bali et al., 1995), but this may be due to an inadvertent inactivation of a developmentally important gene by an additional, random insertion of the targeting vector (Niswender et al., 1997a).

Transgenic rescue of GK-deficiency by β-cell-specific expression of mouse GK cDNA is a complete success in the case of $GK^{+/-}$ mice, but only partial in the case of $GK^{-/-}$ mice since, while hepatic histopathology and circulating lipids are fully restored, some 50 % of the rescued $GK^{-/-}$ mice remain clearly glucose intolerant (Grupe et al., 1995). However, complete rescue of $GK^{-/-}$ mice can be achieved by transgenic expression of the 83 kb mouse GK genomic fragment (Niswender et al., 1997a).

To more clearly define the role of GK-deficiency in pancreatic β-cells in MODY2, β-cell-specific GK ($GK^{\beta}$) knock-out mice have been generated, either by disrupting the β-cell-specific first exon of the GK gene (Terauchi et al., 1995), or by using β-cell-specific Cre-lox system (Postic et al., 1999). Similarly to the "whole-body" GK knock-out, also $GK^{\beta}$-null mice develop severe diabetes shortly after birth and die, with few exceptions, within the first post-natal week (Postic et al., 1999; Terauchi et al., 1995), apparently from dehydration associated with the marked glucosuria (Terauchi et al., 1995). Since $GK^{\beta}$-null pups have normal body weight and blood glucose at the time of birth, and their pancreata appear normal, β-cell GK is not essential for normal development, pancreatic differentiation, or insulin biosynthesis (Terauchi et al., 1995).

Heterozygous $GK^{\beta}$-null ($GK^{\beta+/-}$) mice have mild diabetes, as demonstrated by the early-onset glucosuria, fasting and fed hyperglycemia, and impaired glucose tolerance (Postic et al., 1999; Terauchi et al., 1995). Furthermore, the rates of insulin secretion, glucose turnover, glycogen synthesis and glucose infusion are decreased by 50 to 70 % during the hyperglycemic clamp (Postic et al., 1999; Terauchi et al., 1995). Insulin sensitivity of $GK^{\beta+/-}$ mice is, however, normal (Terauchi et al., 1995). Interestingly, the diabetic phenotype of $GK^{\beta+/-}$ mice with ~50 % GK activity in β-cells is worse than that in mice where only ~30 to 50 % of β-cell GK activity survives inactivation by GK ribozyme or GK antisense mRNA (Efrat et al., 1994; Ishihara et al., 1995). The minor differences between the phenotypes of these two types of genetic GK knock-out models may arise from different genetic backgrounds, or the residual GK activity in mice generated utilizing the Cre-lox system (Postic et al., 1999). Additionally, in mice lacking the first β-cell-specific GK exon (Terauchi et al., 1995), GK-deficiency may not be limited to β-cells, but extends also to the probable glucose-sensing units in certain cells in the brain and gut.

Detailed studies on $GK^{\beta+/-}$ islets have revealed that β-cells with partial GK-deficiency secrete insulin abnormally so that insulin sensitivity is impaired but normal glucose response is retained (Aizawa et al., 1996; Terauchi et al., 1995). $GK^{\beta}$-null β-cells secrete some insulin, presumably due to a low activity of other HKs, but the response to glucose stimulation is absent (Terauchi et

al., 1995). These data demonstrate the vital role of GK as a glucose sensor for insulin secretion in pancreatic β-cells. However, GK is not absolutely required for insulin secretion in response to certain non-glucose segretagogues (Terauchi et al., 1995).

Interplay between insulin resistance and impaired insulin secretion in the development of type 2 diabetes has been studied by breeding $GK^{\beta+/-}$ mice with IRS1-null mice, which have normal glucose tolerance, but are insulin resistant and hyperinsulinemic (Terauchi et al., 1997). The resulting double knock-out mouse model presents a genetic reconstitution of diabetes as a polygenic disease, because neither defect alone causes type 2 diabetes, but the $GK^{\beta+/-}$ x IRS1-null mice become overtly diabetic (Terauchi et al., 1997). Specific phenotypic features of $GK^{\beta+/-}$ x IRS1-null mice include fasting hyperglycemia and hyperinsulinemia (2 and 1.5-fold, respectively, *vs.* wild type), markedly impaired glucose tolerance (worse than in $GK^{\beta+/-}$ mice) and insulin sensitivity (worse than in IRS1-null mice), and increased β-cell mass (equal to IRS1-null mice). To complete the model, the development of exaggerated glucose intolerance in double knock-outs is age-dependent, possibly due to β-cell exhaustion (Terauchi et al., 1997).

In contrast to lethal phenotype of $GK^{\beta}$-null mice, almost total inactivation of hepatocytic GK ($GK^{L}$-null) by utilizing Cre-lox technique is not life-threatening (Postic et al., 1999). Adult $GK^{L}$-null mice have increased fed plasma insulin levels (2-fold), but only ~10 % increase in fed blood glucose, and no changes in plasma FFA, triglycerides or β-hydroxybutyrate, or in hepatic or muscle glycogen content. Fasting eliminates hyperinsulinemia in $GK^{L}$-null mice, but exacerbates hyperglycemia (1.4-fold). Results from the hyperglycemic clamp reveal marked decreases in the rates of glucose turnover and infusion (60 to 70 %), and in net hepatic glycogen synthesis (90 %). Despite fed hyperinsulinemia, a hyperglycemic clamp resulted in a surprising 70 % fall in plasma insulin in $GK^{L}$-null mice (Postic et al., 1999). While leaving several questions open for further studies, these results emphasize the importance of hepatic GK in glycogen storage during hyperglycemia. They also suggest that in the basal state an alternative, GK-independent pathway is active in the liver, providing substrate for glycogen synthesis (Postic et al., 1999).

In summary, studies with mice over- or underexpressing different levels of GK show a gene dose-dependency in the effects of GK on glucose metabolism. Pancreatic β-cell-GK has been shown to have a significant role in the maintenance of whole-body glucose homeostasis by sensing blood glucose levels and regulating insulin secretion whereas hepatic GK participates in the regulation of glucose homeostasis mainly by controlling glycogen storage (Postic et al., 2001).

### 2.4.6 Overexpression of hexokinase II in striated muscle

Chang et al. have used rat muscle creatine kinase promoter-enhancer elements to overexpress human HKII cDNA specifically in mouse striated muscle (Chang et al., 1996). The resulting increase in HKII mRNA and enzyme activity varies from muscle to muscle, but is ~5-fold on average compared with control, corresponding to levels achieved in other studies by *in vivo* hyperinsulinemia (Mandarino et al., 1995; Postic et al., 1993) or exercise (Hildebrandt et al., 2003; Koval et al., 1998; Nordsborg et al., 2003; O'Doherty et al., 1996; O'Doherty et al., 1994; Pilegaard et al., 2000). In the basal state, overexpression of HKII does not affect subcellular distribution of muscle HKII, nor does it have statistically significant effects on systemic glucose tolerance or insulin responsiveness, muscle glycogen content, or plasma levels of glucose, insulin, or lactate. However, in the hyperglycemic-hyperinsulinemic condition the level of G6P, the product of HK enzyme activity, is increased in skeletal muscle by 44 %, and a concurrent decrease in blood glucose level occurs. Furthermore, both in basal and insulin-stimulated state, HKII overexpression increases glucose uptake into isolated skeletal muscles. Thus, phosphorylation can be seen important in determining the rate of muscle glucose uptake (Chang et al., 1996).

To elaborate the consequences of HKII overexpression further *in vivo*, the same HKII overexpressing mice as reported in (Chang et al., 1996) have been subjected to the euglycemic-hyperinsulinemic clamp and exercise experiments (Halseth et al., 1999). Again, overexpression of HKII did not have an effect in the basal state, further strengthening the view that glucose transport plays a limiting role in basal muscle glucose uptake. However, glucose infusion rate during the euglycemic-hyperinsulinemic clamp was increased in HKII transgenic mice, indicating increased whole body glucose metabolism, presumably due to an increase in stimulated muscle glucose uptake. Furthermore, moderate-intensity exercise resulted in increased muscle glucose uptake, especially after prolonged (18 h) fast when glycogen stores are somewhat depleted, thereby limiting the product inhibition of HKII by G6P originating from glycogenolysis. Thus, in conditions where glucose utilization is increased by insulin stimulation or exercise, phosphorylation was concluded to exert more control over muscle glucose uptake than transport (Halseth et al., 1999).

Glucose phosphorylation becomes rate-limiting in muscle glucose disposal in mice overexpressing GLUT1 because the natural downward glucose gradient between the extra- and intracellular space is nearly equalized by the dramatic increase in basal glucose uptake (Ren et al., 1993). Thus, it has been hypothesized that muscle-specific overexpression of HKII in addition to GLUT1 overexpression might further increase muscle glucose uptake and whole-

body glucose disposal (Hansen et al., 2000). However, in GLUT1/HKII mice, glucose transport into isolated muscles is unaltered, as are both basal and insulin-stimulated whole-body glucose disposal, despite strikingly increased steady-state G6P (3.2-fold) and glycogen (7.5-fold) levels. These surprising results, partly in contrast to (Chang et al., 1996), were thought to result primarily from the inhibition of *de novo* glycogen synthesis by the very high levels of steady-state muscle glycogen (Hansen et al., 2000).

The effects of a simultaneous increase in both glucose transport and phosphorylation has been studied by crossing FVB/NJ mice overexpressing both GLUT4 (Leturque et al., 1996) and HKII (Chang et al., 1996) in muscle-specific manner (Lombardi et al., 1997). Results from the hyperglycemic-hyperinsulinemic clamp and experiments with isolated soleus muscles suggest that in GLUT4/HKII double-transgenic mice, glucose phosphorylation is rate limiting in skeletal muscle in the case of hyperglycemia, but not in the case of hyperinsulinemia (Lombardi et al., 1997).

Very recently results have been presented for an elegant study designed to discern the distribution of control for muscle glucose uptake *in vivo* during rest and moderate-intensity exercise (Fueger et al., 2003). Muscle glucose uptake was investigated in mice overexpressing either GLUT4 (Olson et al., 1993) or HKII (Chang et al., 1996), or both. The hypothesis was that if a protein catalyzing a given metabolic step is rate-limiting either at rest or during exercise, overexpression of that protein will result in increased muscle glucose uptake in that condition. Indeed, it was demonstrated that glucose transport is rate-limiting for muscle glucose uptake in the sedentary state, but glucose phosphorylation is in control during exercise. It was also shown that when both transport and phosphorylation barriers are lowered, *i.e.* both GLUT4 and HKII are overexpressed, glucose delivery from blood becomes rate-limiting. These data strongly suggest that the control of glucose utilization in skeletal muscle can shift between glucose delivery, transport and phosphorylation as required by the alternating physiological states (Fueger et al., 2003).

**Table 2.** Summary of the main phenotypic effects of genetically altered glucose transport and phosphorylation in mice.

| Gene | Alteration | Tissues | Principal phenotypic features |
|---|---|---|---|
| GLUT1 | Overexpression | Skeletal muscle | Glc ↓, Ins ↔, GT ↑, basal MGU ↑, whole-body glucose utilization ↑ |
| | | Heart | Cardiac MGU and glycolysis ↑, protection against the fatal effects of cardiac hypertrophy |
| GLUT2 | Knock-out | All | -/-: Type 2-like diabetes and early death, severe glucosuria, and impaired insulin secretion (mostly rescued by β-cell GLUT1 or GLUT2-tg, except: fed Ins ↓ and glucagon ↑, and due to persisting glucosuria, fasted Glc ↓) |
| GLUT4 | Overexpression | Natural pattern | Glc ↓, Ins ↓, GT ↑↑, muscle glycogen ↑↑, parameters of hyperinsulinemic clamp ↑, HGO ↔ |
| | | Skeletal muscle | Mostly as above; the changes in Glc and Ins are age-dependent |
| | | Adipose tissue | Glc ↓, GT ↑, glucose transport into adipose tissue ↑↑↑, and adipocyte number ↑, but size ↔ |
| | Knock-out | All | +/-: insulin resistance (not hepatic) and diabetes in males; hypertension, and cardiac and adipose cell size ↑<br>-/-: no diabetes or impaired GT, but Ins ↑, IS ↓, basal MGU and GS ↑, adiposity ↓↓ , cardiac cell size ↑, longevity ↓<br>(complete rescue of both $GLUT4^{+/-}$ and $GLUT4^{-/-}$ mice by skeletal muscle GLUT4-tg) |
| | | Skeletal muscle | -/-: Similar diabetes than in $GLUT4^{+/-}$ mice, but with hepatic insulin resistance |
| | | Heart | -/-: Mild cardiac pathologies |
| | | Adipose tissue | -/-: IS of adipocyte glucose uptake ↓, and insulin resistance in the liver and skeletal muscle |
| HKB | Overexpression | β-cells | Glc ↓, Ins ↑, insulin secretion ↑; suggestion that GK serves as a glucose sensor for β-cells |
| | | Heart | Cardiac glycolysis and glycogen storage ↑ |
| GK | Overexpression | Natural pattern | Glc ↓, Ins ↔, GT ↑, IS ↑, hepatic glucose metabolism ↑, insulin secretion ↔ |
| | | Liver | Glc ↓, Ins ↓, GT ↑, hepatic glycogen accumulation ↑ |
| | | Skeletal muscle | Protection from the adverse effects of experimental diabetes |
| | Knock-out | All | +/-: Glc ↑, GT ↓, relative insulin secretion ↓, and abnormal liver glucose metabolism (complete rescue by β-cell GK-tg)<br>-/-: Severe diabetes: Glc ↑↑↑, Ins ↓↓, lipids ↑↑, and early death (partial rescue by β-cell GK-tg) |
| | | β-cells | +/-: Mild diabetes: Glc ↑, GT ↓, IS ↔, parameters of hyperinsulinemic clamp ↓, and glucosuria<br>-/-: Similar to GK-deficiency of all tissues |
| | | Liver | -/-: Fasted Glc ↑ and Ins ↔, lipids ↔, liver and muscle glycogen ↔, parameters of hyperinsulinemic clamp ↓ |
| HKII | Overexpression | Skeletal muscle | Glc, Ins, GT, IS, and muscle glycogen ↔, but MGU ↑ (*in vivo* and *in vitro*, and in the basal, and insulin and exercise-stimulated state), and during hyperinsulinemic clamp, whole-body glucose metabolism ↑ |

For further details and references, see text. Glc, plasma glucose; Ins, plasma insulin; GT, glucose tolerance; MGU, muscle glucose uptake; tg, transgene; HGO, hepatic glucose output; IS, insulin sensitivity; GS, muscle glycogen synthesis.

# 3 AIMS OF THE STUDY

The main purpose of this study was to elucidate the role of HKII in glucose metabolism using a HKII knock-out mouse model.

Specific aims of this thesis were

1. To determine the nucleotide sequence and structural characteristics of the mouse HKII gene and cDNA and the expression pattern of mRNA. (**I**)

2. To generate and characterize a mouse line deficient in HKII, with emphasis on developmental consequences and glucose metabolism. (**II**)

3. To determine the role of glucose phosphorylation in the control of muscle glucose uptake in the basal state and during exercise. (**III**)

4. To further characterize the phenotype of mice heterozygous for HKII deficiency, with emphasis on altered states of glucose metabolism. (**IV**)

# 4 MATERIALS AND METHODS

## 4.1 Animals

Mice were kept at the Laboratory Animal Centres of the University of Kuopio (**II**, **IV**), or the Vanderbilt University (**III**), on 12 h day/night cycle, and fed regular low-fat chow (4 % fat), or in the high-fat feeding experiment (**II**) Western type diet (42 % of calories from fat; Harlan Teklad, Madison, WI, USA) for 16 to 20 weeks. Experimental $HKII^{+/-}$ mice were backcrossed at least three generations to a BALB/c x DBA/2 genetic background (**II-IV**). When anesthesia was used, it was induced by fentanyl-fluanisone (Janssen Pharmaceutical, Beerse, Belgium) and midazolam (Hoffman-LaRoche, Basel, Switzerland) (**II**, **IV**) or by sodium pentobarbital (**III**). Housing and procedures involving experimental animals were performed in accordance with protocols approved by the Department for Social and Health Services, the State Provincial Office of Eastern Finland, Kuopio, Finland, or the Institutional Animal Care and Use Committee of the University of Kuopio, Finland, or the Vanderbilt University Animal Care and Use Committee, Nashville, Tennessee, USA.

## 4.2 Molecular biology methods

### *4.2.1 Isolation of the mouse HKII gene and cDNA (I)*

A 129/SvJ mouse genomic library and a mouse oligo-dT primed diaphragm cDNA library (Stratagene Inc., La Jolla, CA, USA) were screened using the entire rat HKII cDNA (kindly provided by Dr. J.E. Wilson, Michigan State University) and PCR-generated fragments of mouse HKII cDNA as probes, as described (**I**). In the case of genomic clone containing exon 2, a PCR based method was used as described (**I**) (Israel, 1993). Phage inserts of isolated positive clones were subcloned, and sequenced in the sequencing core facility at the A.I.Virtanen Institute for Molecular Sciences, University of Kuopio, Finland.

To generate a virtually full-length mouse HKII cDNA, the sequences (1.6 kb from the 5' end) lacking from the largest, yet incomplete, cDNA clone were amplified by RT-PCR, sequenced, and cloned into the plasmid containing the incomplete cDNA as described (**I**).

### *4.2.2 Nucleotide and amino acid sequence data analysis (I)*

Sequence assembly was performed using GeneSkipper (EMBL, Heidelberg, Germany). Putative binding sites for transcription factors were localized using "Transcription Element Search Software" utilizing Transfac v3.3 database at http://www.cbil.upenn.edu/tess/. Repeat elements

were identified by RepeatMasker2 at http://ftp.genome.washington.edu/RM/RepeatMasker.html. Sequences were aligned with ClustalX 1.5b (Thompson et al., 1994).

### 4.2.3 Northern blot analysis (I, II)

Total RNA was isolated by the acidic guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). To perform a Northern blot analysis, 15-20 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 HKII cDNA probe encompassing exon 9 (**I**) or exons 11 to 13 (**II**). Equal loading and RNA quality of the samples was verified by staining the blot with methylene blue. For additional verification of the quality of RNA (**I**), the blot was probed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The blots were visualized on autoradiography film. HKII mRNA expression levels were determined from scanned images of the blots (**II**).

### 4.2.4 Rapid amplification of mouse HKII cDNA ends (I)

Total RNA from tissues of a 7 week old male BALB/c x DBA/2 mouse were used as a template in rapid amplification of cDNA ends (RACE). Two 5'- and one 3'-RACE experiments were performed with 5'/3' RACE kit (Boehringer-Mannheim, Mannheim, Germany) essentially according to the manufacturer's protocols, and as described (**I**). One 5'-RACE was designed to reveal the transcription initiation site of the mouse HKII gene in adipose tissue, heart, skeletal muscle and testis, and the other to reveal the presence of splice variants involving 5'-UTR and exon 1 in the same tissues. The 3'-RACE was performed to verify the primary polyadenylation site, suggested by sequence analysis, in skeletal muscle and testis.

### 4.2.5 Generation of HKII-deficient mice (II)

#### 4.2.5.1 Construction of the targeting vector

The region of HKII enzyme encoded by exon 4 and onwards, shown to contain glucose and ATP binding sites (Wilson, 1995), was included in the targeting vector (pHK2Neo), which was constructed as follows. Two DNA sequences containing mouse HKII exon 4 (XbaI fragment) and exons 5-10 (XbaI-XhoI fragment) were subcloned to pUC19 cloning vector, yielding p8X4 and p2XS1, respectively. The mouse HKII gene was disrupted by inserting a positive selection cassette from pTV-0 (gift from Dr. H. van der Putten), including a gene for neomycin phosphotransferase (neo) driven by herpes simplex virus thymidine kinase promoter, into a BsmI site of exon 4 in p8X4 via an oligonucleotide linker containing a XhoI site, yielding p8X4Neo.

The XbaI fragment of p8X4Neo was then inserted into XbaI site of p2XS1, yielding the final targeting vector, pHK2Neo (Fig. 3), having 0.6 kb 5' and 9.2 kb 3' homology arms.

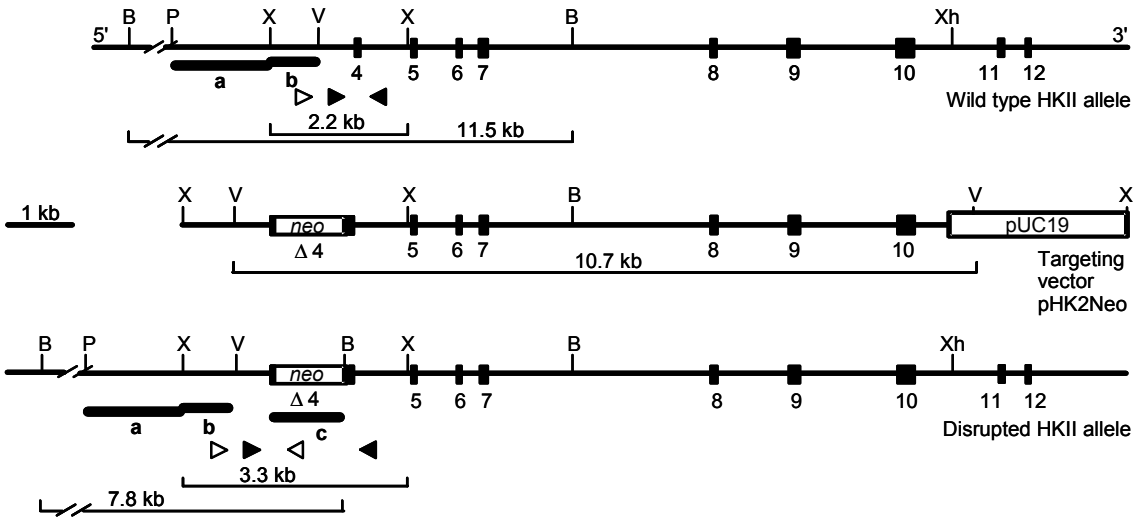



**Figure 3.** Disruption of the mouse HKII gene by homologous recombination. Structures of the wild-type HKII allele, targeting vector pHK2Neo and the disrupted HKII allele are schematically presented. Exons (black boxes) and selected restriction sites (B, BamHI; P, PstI; V, VspI; X, XbaI and Xh, XhoI) are indicated. Targeting vector contains a neo gene to allow the selection of homologous recombination events and to disrupt the coding region of the HKII gene in exon 4. The 10.7 kb VspI fragment electroporated into embryonic stem cells is indicated under the targeting vector. Southern analysis probes (a, b and c) used in this study are depicted as black bars. PCR primers for identification of the targeted ES cells and knock-out mice were HK2KO-F1 (open forward arrowhead), Neo-R1 (open reverse arrowhead), HK2KO-F2 (solid forward arrowhead), and HK2KO-R1 (solid reverse arrowhead).

#### *4.2.5.2 Targeting of the HKII gene in embryonic stem cells*

Thirty micrograms of agarose gel purified targeting construct was introduced to 129/Ola mouse E14 embryonic stem cells (Hooper et al., 1987) by electroporation (Gene Pulser, BioRad Life Science Group, Hercules, CA) as described (**II**) (Joyner, 1993). The cell clones surviving neomycin selection were analyzed as described (**II**) to screen for the correctly targeted HKII gene. Shortly, PCR with forward primer external to the targeting construct (HK2KO-F1: 5'-AACCACGACGCCCAATGATTTAG-3') and reverse primer internal to neo (Neo-R1: 5'-GTGCCCAGTCATAGCCGAATAGC-3') was performed. Positive clones were then analyzed by Southern blot with probes for neo and, as depicted in Fig. 3, for sequences external to the targeting construct.

#### *4.2.5.3 Generation of chimeric mice*

ES cells carrying the correctly targeted HKII gene were aggregated with BALB/c x DBA/2 morulae (Wood et al., 1993) and transferred into pseudopregnant BALB/c x DBA/2 foster mothers. The resulting chimeric males, identified by the presence of agouti coat pigmentation, were bred to BALB/c x DBA/2 females to establish germline transmission of the disrupted

allele, and to dilute the 129/Ola genetic background originating from the embryonic stem cells. The offspring carrying disrupted HKII alleles were identified by PCR as described (below; **II**).

### *4.2.6 Detection of the disrupted HKII allele (II-IV)*

To facilitate convenient detection of both wild-type and disrupted HKII alleles in one PCR reaction, an allele discriminating PCR scheme was developed as described (**II**), including a common forward primer, specific for a sequence 5' of exon 4 (HK2KO-F2: 5'-ACTCTCCTGCCGCCCTGC-3'), and two allele specific reverse primers, one for neo (disrupted allele) (Neo-R1) and one for intron 4 (wild-type allele) (HK2KO-R1: 5'-CCCCTCATCGCCACCGC-3') (Fig. 3).

### *4.2.7 Quantitative analysis of gene expression (IV)*

To study the expression of various genes, encoding key glycolytic enzymes and insulin signalling proteins, in the sedentary state and after exercise, wild-type and $HKII^{+/-}$ female mice (n = 4-6 per group) were subjected to a 60 min protocol where they either ran on a flat-bed treadmill (4 % grade, 15 cm/s speed), or remained sedentary. After 6-h recovery period, mice were killed by cervical dislocation and the tissues were rapidly excised. Total RNA from quadriceps muscle was extracted and processed to cDNA as described (**IV**).

Quantitative RT-PCRs were performed using 3.75, 15 or 60 ng (RNA equivalents) of template, gene specific primers, and SYBR Green Master Mix from Applied Biosystems in TaqMan 7700 (Applied Biosystems, Foster City, CA, USA). Running conditions were 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60 or 63°C. Primer sequences and other details are given in Table 3. Data were normalized to beta-actin mRNA expression. Sedentary mRNA expression of selected genes was analyzed also in additional quadriceps samples from female wild-type and $HKII^{+/-}$ mice (n = 5 and 5, respectively), and the results were pooled with the original sedentary groups.

## 4.3 Characterization of embryonic lethality of $HKII^{-/-}$ mice

### *4.3.1 Genotyping of early mouse embryos by PCR (II)*

Pups homozygous for the HKII knock-out allele ($HKII^{-/-}$) were not detected within the offspring of $HKII^{+/-}$ x $HKII^{+/-}$ matings. Thus, embryonic lethality due to complete lack of HKII was suspected. To determine the developmental stage where $HKII^{-/-}$ embryos die, $HKII^{+/-}$ mice were mated and the embryos at selected stages were analyzed by allele discriminating PCR as described (**II**).

**Table 3.** Parameters for quantitative PCR assays.

| Gene | Gene product | Primers | | | Product | | |
|---|---|---|---|---|---|---|---|
| | | GenBank Accession | Forward primer | Reverse primer | bp | $T_a$ [1] | 5'-end [2] |
| Actb | β-Actin | NM_007393 | caaccgtgaaaagatgacccaga | acgaccagaggcatacagggac | 100 | 63 | 422 |
| Hk2 | Hexokinase II | NM_013820 | cgcccctgtgaagatgttgc | ccccttcgcttgccattacg | 138 | 60 | 1997 |
| Glut4 | Glucose transporter 4 | NM_009204 | gctgtgcttggctcccttca | gggtgccttgtgggatggaa | 139 | 63 | 250 |
| Gpi1 | Glucose 6-phosphate isomerase 1 | NM_008155 | gcactttgtcgccctgtctacg | gccacctacccaatcccagaac | 94 | 60 | 775 |
| Pfkm | Phosphofructokinase, muscle type | NM_021514 | acgggaagccaatcacctcag | ttccacacccatcctgctgc | 149 | 60 | 851 |
| Gapd | Glyceraldehyde 3-phosphate dehydrogenase | NM_008084 | gcagcccagaacatcatccct | tgaccttgcccacagccttg | 58 | 63 | 647 |
| Pgm1 | Phosphoglucomutase | NM_028132 | tttgatggtgacggggatcg | cggaatgctgaagatgttggc | 105 | 60 | 899 |
| Gys1 | Glycogen synthase 1, muscle type | NM_030678 | aacaagatgctggacaaggaggac | tgggaaagactgccgctga | 75 | 60 | 1301 |
| Gsk3b | Glycogen synthase kinase 3 β | NM_019827 | ccgaggagagcccaatgtttc | gcaacaattcagccaacacacag | 131 | 63 | 790 |
| Pygm | Glycogen phosphorylase | NM_011224 | tccgcacacagcagcattactac | catcacaggcgttctccaagg | 126 | 60 | 256 |
| G6pd | Glucose 6-phosphate dehydrogenase | NM_008062 | gacgacatccgaaagcagagtga | ttacgggcaaagaactcctcca | 83 | 60 | 284 |
| Pkm2 | Pyruvate kinase, muscle type | NM_011099 | cctctggaggctgttcgcat | cggcggagttcctcgaatag | 89 | 63 | 1117 |
| Pdha1 | Pyruvate dehydrogenase E1 α1 | NM_008810 | agcagcagccagcacggacta | tgcctctcggacgcacaagat | 90 | 60 | 816 |
| Pck1 | Phosphoenolpyruvate carboxykinase 1 | NM_011044 | gctggatgtcggaagaggactt | ggcgagtctgtcagttcaatacca | 148 | 60 | 484 |
| Ldh1 | Lactate dehydrogenase 1, A chain | NM_010699 | cagaactgggcactgacgca | cctcgtaggcactgtccacca | 75 | 63 | 845 |
| Irs1 | Insulin receptor substrate 1 | X69722 | tgggtccagcagcagtagca | gtttggcatgaggaagggca | 113 | 60 | 2491 |
| Pi3kca | Phosphatidylinositol 3-kinase, catalytic subunit α | NM_008839 | cgggaacgtgtgccatttg | atgctgccgaattgctaggtaag | 138 | 60 | 2845 |
| Pi3kra | Phosphatidylinositol 3-kinase, regulatory subunit α | U50413 | aggtgtgcggcagaagaagc | tggtggggcaaatcctcatc | 96 | 60 | 2335 |
| Pparg | Peroxisome proliferator-activated receptor γ 1 and 2 | NM_011146 | aggcgagggcgatcttgaca | gcagggggtgatatgtttgaact | 117 | 60 | 818 |

[1] Annealing temperature in the PCR cycle in °C. [2] The nucleotide in the GenBank Accession corresponding to the 5'-end of the PCR product.

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 1x phosphate buffered saline (PBS) supplemented with 1 mg/ml BSA, transferred individually to 3 µl of 1xPCR buffer containing 50 µg/ml proteinase K, lysed for 2 h at 55°C, and subjected to the allele discriminating PCR. To analyze gastrulae, embryos were dissected at 7.5 and 8.5 days post coitum from pregnant mothers in 1xPBS, photographed with a camera attached to Axiovert 135M or 35M microscope (magnification 50-400 x), and genotyped similarly to blastocysts.

### *4.3.2 Histological analysis*

$HKII^{+/-}$ mice were mated and the decidua holding the embryos were dissected at selected time points (days 6.5 – 8.5) post coitum from pregnant mothers in 1xPBS, fixed in 4 % paraformaldehyde for 4 h in 4°C, and stored in 1xPBS containing 15 % sucrose. The decidua were embedded in paraffin and serially sectioned (8 µm). To genotype the embryos, duplicate sections per embryo were scraped clear of paraffin-embedded decidua with 18G needle under preparative microscope, and the embryonic parts were transferred into 15 µl of lysis buffer (1xPCR buffer, 200 µg/ml proteinase K). Paraffin was melted by incubating the samples in 65°C water bath for 10 min, and the tissues lysed overnight in 37°C cabinet. Finally, wild-type HKII and neo alleles were separately detected by PCR with manual hot start under standard conditions using 5 µl of the lysate as template, and the appropriate primers for the allele discriminating PCR as described (**II**). Selected sections of the genotyped embryos in 50-75 µm intervals were stained with hematoxylin and eosine for microscopic study and photography.

## 4.4 Assay of HKI and HKII enzyme activities (II, IV)

Soluble HKI and HKII enzyme activities were analyzed either by a conventional assay, performed one sample at a time, as described (**II**), or by a modified, semi-high-throughput assay, up to 24 samples in duplicate at a time (**IV**). Shortly, for both types of assays, tissues (**II**: hind limb muscle, heart and adipose tissue; **IV**: hind limb muscle) were homogenized, and centrifuged to obtain a supernatant free of cellular debris. One aliquot of the supernatant was assayed for total HK activity. Another aliquot was heat-treated at 45°C for 1 h and assayed for the heat stable hexokinase activity, mainly HKI (Grossbard and Schimke, 1966; Wilson, 1998). HK activities were determined by measuring the formation of coenzyme NADPH in a coupled enzyme assay system containing G6P dehydrogenase from *Leuconostoc mesenteroides* (Sigma, St. Louis, MO, USA), and expressed as milliunits per minute per mg protein, where 1 unit equals to the amount of enzyme activity forming 1 µM of NADPH in 1 minute at 23°C and pH 8.0.

## 4.5 Metabolic studies of HKII$^{+/-}$ mice (II - IV)

### *4.5.1 Analytical methods*

Plasma glucose concentrations were determined either by Glucose & Lactate Analyzer 2300 Stat Plus (YSI, Yellow Springs, OH, USA) (**II, IV**), Precision Q.I.D™ blood glucose sensor with Precision Plus electrodes (Medisense, Abbott Laboratories Ltd., Birmingham, UK) (**II**), HemoCue Glucose Test System (Mission Viejo, CA, USA) (**III**), or microfluorometrically (Passonneau and Lowry, 1993) (**IV**). Plasma insulin levels were determined using a rat insulin ELISA kit (Crystal Chem Inc., Chicago, IL, USA) with mouse insulin as a standard (**II, IV**), or by double antibody method (Morgan and Lazarow, 1965) (**III**). Plasma triglyceride, total cholesterol, and non-esterified fatty acid concentrations were assayed with kits purchased from Roche Molecular Biochemicals, Boehringer-Mannheim or Wako Chemicals.

### *4.5.2 Glucose and insulin tolerance tests*

Glucose tolerance and insulin sensitivity of wild-type and HKII$^{+/-}$ mice were analyzed by intraperitoneal glucose (GTT) and insulin (ITT) tolerance tests. Blood samples were collected either from the saphenous or the tail vein. Specific parameters for tests performed at various physiological states are given in Table 4.

### *4.5.3 Glucose uptake during exercise (III)*

#### *4.5.3.1 Surgical procedures*

The surgical procedures utilized were similar to those described previously (Halseth et al., 1999; Niswender et al., 1997b; Rottman et al., 2002). Mice were anesthetized with pentobarbital (70 mg/kg body weight). The left common carotid artery was catheterized for sampling of arterial blood with a two-part catheter consisting of PE-10 (inserted into the artery) and Silastic (0.025 in OD). The right jugular vein was catheterized for infusions with a Silastic catheter (0.025 in OD). The free ends of catheters were tunneled under the skin to the back of the neck, where they were attached via stainless steel connectors to lines made of Micro-Renathane (0.033 in OD), which were exteriorized and sealed with stainless steel plugs. Lines were kept patent by flushing each daily with 10-40 µl saline containing 200 U/ml of heparin and 5 mg/ml of ampicillin. Animals were individually housed after surgery and body weight was recorded daily. Following an ~5 day period in which body weight was restored (within 10 % of pre-surgery body weight) mice were acclimated to treadmill running with a single 10-min bout of exercise (0.5-0.6 mph, 0 % grade). Experiments were performed two days following the treadmill acclimation trial.

**Table 4.** Metabolic studies of wild-type and $HKII^{+/-}$ mice at various physiological states.

| Study | Physiological state | Test | Dose[1] | Anes-thesia | Age[2] (months) | Sex | n | Diet | Fast (hours) | Time points (minutes) | Blood assays[3] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **II** | Sedentary | ITT | 0.75 mU/g | + | 4 | F | 5 | Chow | 6 | 0, 15, 30, 60 | Glc |
| | | GTT | 1.0 mg/g | + | 3 | M | 10 | Chow | 12 | 0, 15, 30, 60 | Glc, Ins |
| | High-fat diet | Plasma assays | - | - | 1, 2, 3 | F, M | 4-6 | Chow/HFD | 4-8 | - | Glc, Ins |
| | | ITT | 0.75 mU/g | + | 4 | | | | 4 | 0, 15, 30, 60 | Glc, Chol, TG |
| | | GTT | 1.0 mg/g | + | 4½ | | | | 4 | 0, 15, 30, 60 | Glc, Ins |
| **III** | Exercise[4] | Glucose uptake | 12 µCi DG | - | 4 | F, M | 8-11 | Chow | 5 | 0, 10, 15, 20, 30 | Glc, Ins, NEFA, Hematocrit, DG |
| **IV** | Aging | Plasma assays | - | - | 22 | F | 10-11 | Chow | 0 / 14 | - | Glc, Ins, Chol, TG |
| | | ITT | 0.75 mU/g | + | 22 | | | | 14 | 0, 20, 40, 80 | Glc |
| | | GTT | 2.0 mg/g | + | 23 | | | | 14 | 0, 20, 40, 80 | Glc |
| | Sedentary | ITT | 0.20 mU/g | - | 4-8 | F, M | 7-15 | Chow | 12 | 0, 20, 40, 80, 120 | Glc |
| | | GTT | 2.0 mg/g | - | 4 | F | 11-13 | Chow | 8 | 0, 15, 120 | Glc, Ins |
| | Exercise[5] | GTT | 2.0 mg/g | - | 4 | F | 10 | Chow | 12 | 0, 15, 30, 60, 90 | Glc |

[1] In ITT, insulin (Actrapid®, Novo Nordisk, Denmark), and in GTT, glucose as 20 % D-glucose solution was administered intraperitoneally to an indicated dose per grams body weight. At the end of an ITT, 0.5 mg/g body weight D-glucose was administered to counter the remaining effects of insulin. For the glucose uptake experiment, 12 µCi [2-$^3$H]-deoxyglucose (DG) (New England Nuclear, Boston, MA) bolus was administered at 5 minutes through chronic catheter.

[2] For the high-fat diet experiment, the pre-diet samples were taken at the first time point for blood assays. This also indicates the starting age of the high-fat diet.

[3] Glc, glucose; Ins, insulin; Chol, total cholesterol; TG, total triglycerides; DG, [2-$^3$H]-deoxyglucose. For the aging experiment, Chol and TG were analyzed only from fasted samples.

[4] Moderate-intensity running, corresponding to ~80 % of $VO_{2max}$ (Fernando et al., 1993), was performed on a motorized treadmill (0 % slope, 0.6 mph (27 cm/s) speed). Common carotid artery and right jugular vein were catheterized for blood sampling and infusions, respectively.

[5] Moderate-intensity running, corresponding to ~75 % of $VO_{2max}$ (Fernando et al., 1993), was performed on a flatbed treadmill (4 % slope, 15 cm/s speed).

#### 4.5.3.2 Experimental procedures

Conscious mice were placed in a 1 l plastic container lined with bedding and fasted for 5 h on the day of the experiment. Approximately 1 h prior to an experiment, Micro-Renathane (0.033 in OD) tubing (22 cm long) was connected to the catheter leads, and mice were placed on an enclosed treadmill and allowed to acclimate to the new environment. At t = 0 min, a baseline blood sample (150 µl) was drawn for the measurement of blood glucose hematocrit, and plasma insulin and non-esterified fatty acids (NEFA; equal to FFA). The remaining red blood cells were washed once with 0.9 % saline containing 10 U/ml of heparin and reinfused. The mice either remained sedentary (n = 8 WT, 11 $HK^{+/-}$) or ran (n = 11 WT, 11 $HK^{+/-}$) on the treadmill for 30 min at 0.6 mph (27 cm/s) with a 0 % grade. The selected work intensity was ~80 % of maximal oxygen consumption (Fernando et al., 1993). At t = 5 min, a 12 µCi bolus of [2-$^3$H] deoxyglucose ([2-$^3$H]DG) (New England Nuclear, Boston, MA) was administered in order to provide an index of tissue-specific glucose uptake, Rg, calculated as previously described (Kraegen et al., 1985). At t = 10, 15, and 20 min, ~50 µl of blood was sampled in order to determine arterial blood glucose and plasma [2-$^3$H]DG. At t = 30 min, a 150 µl blood sample was withdrawn in order to determine blood glucose, hematocrit, and plasma insulin, [2-$^3$H]DG, and NEFAs, and mice were anesthetized with an arterial infusion of sodium pentobarbital. The heart, and soleus, gastrocnemius and superficial vastus lateralis (SVL) skeletal muscles were excised, immediately frozen in liquid nitrogen, and stored at -70°C until future tissue analysis.

#### 4.5.3.3 Processing of plasma and muscle samples

Following deproteinization with $Ba(OH)_2$ (0.3 N) and $ZnSO_4$ (0.3 N), [2-$^3$H]DG radioactivity of plasma was determined by liquid scintillation counting (Packard TRI-CARB 2900TR, Packard, Meriden, CT) with Ultima Gold (Packard) as scintillant. Muscle samples were weighed and homogenized in 0.5 % perchloric acid. Homogenates were centrifuged and neutralized with KOH. One aliquot was counted directly in order to determine total radioactivity ([2-$^3$H]DG and phosphorylated [2-$^3$H]DG ([2-$^3$H]DGP)). A second aliquot was treated with $Ba(OH)_2$ and $ZnSO_4$ in order to remove [2-$^3$H]DGP and any tracer incorporated into glycogen and then counted to determine [2-$^3$H]DG radioactivity (Meszaros et al., 1987). [2-$^3$H]DGP is the difference between the two aliquots. In all experiments the accumulation of [2-$^3$H]DGP was normalized to tissue weight.

Muscle glycogen was determined by the method of Chan and Exton (Chan and Exton, 1976) on the heart and contralateral gastrocnemius and SVL muscles. Soleus glycogen was not determined since both of the small muscles were used for the radioactivity assay. Glycogen breakdown in

response to exercise was calculated as the average glycogen concentration for sedentary mice within a genotype minus the individual post-exercise glycogen concentrations. Following deproteinization with 0.5 % perchloric acid, tissue glucose and G6P were measured enzymatically (Lloyd et al., 1978) and expressed as mmol per liter tissue water.

## 4.6 Statistical analyses

Statistical analyses were performed with the SPSS for Windows programs (SPSS Inc., Chicago, IL, USA) using Student's t test for independent samples, Chi-square, Mann-Whitney U, or the analysis of variance, when appropriate. In Study II, glucose and insulin values for the glucose tolerance test in the sedentary state were analyzed by linear regression method and adjusted for the difference in fasting weight between the groups prior to comparison with Student's t test. Insulin levels were logarithmically (ln) transformed for statistical analyses. Data are shown as mean ± SEM. P-value less than 0.05 was considered statistically significant.

# 5 RESULTS

## 5.1 Mouse HKII gene, mRNA and cDNA (I)

### *5.1.1 Gene*

Altogether, 35 kb of mouse HKII gene was sequenced from subclones of six originally isolated mouse 129/SvJ genomic library phage clones (Fig. 4A). Sequence for the promoter region (EMBL access number Y11666) is 6.15 kb in length, comprising 4.8 kb of the promoter, all of exon 1 and 0.8 kb of intron 1. Exon 2, 163 bp in length, is flanked by large introns, and was thus sequenced separately with only short intronic regions (404 bp in total) (Y11667). The third, 28.6 kb sequence (Y11668) comprises exons 3 to 18 with entire introns 3 to 17 as well as a 3.0 kb segment of intron 2, and 1.2 kb of 3' flanking area of the gene. Currently available genetic data from the mouse genome projects shows that the mouse HKII gene locates on region C3 of chromosome 6 where it spans approximately 55 kb of which 46.7 kb contains the coding sequences. Mouse HKII gene contains several longer as well as simple repeats (**I**, Fig. 1A). The exon-intron –structure (Fig. 4A) of the mouse HKII gene is comparable to that of rat and human, and the introns follow the 5'-GT – 3'-AG rule (**I**, Table 2). Similarity of coding and amino acid sequences to those of rat and human HKII genes is considerable: 94.9 and 99.1 %, respectively, for rat, and 87.3 and 94.1 % for human. The N- and C-terminal halves within the mouse HKII protein are also highly conserved, as demonstrated by the 58.9 % sequence similarity.

The proximal, GC-rich promoter region of the mouse HKII gene shares high sequence similarity with rat and human HKII promoters (**I**, Fig. 2). Also the majority of putative transcription factor binding sites within the HKII promoter, including TATA-like box at -30, CREB at -71, CCAAT-box at -86, and an inverted CCAAT-box (Y-box) at -144 bp relative to mouse transcription start site are conserved in the mouse, rat and human. One of the seven putative binding sites for upstream stimulatory factor (USF), sometimes called E-boxes, within the sequenced mouse HKII promoter resides at the proximal, highly conserved region, at -412 bp. In addition, one putative binding site for CCAAT/enhancer binding protein (C/EBP) at -360 bp is shared by the mouse, rat and human HKII promoters while another, at -515 bp, is shared only by the mouse and rat.

### *5.1.2 mRNA*

A significant amount of the major 5.5 kb HKII mRNA, transcribed using the functionally verified primary polyadenylation site, was expressed in mouse heart, skeletal muscle and white adipose tissue (**I**, Fig. 3) with barely detectable expression in lungs, spleen, ovaries and testes.

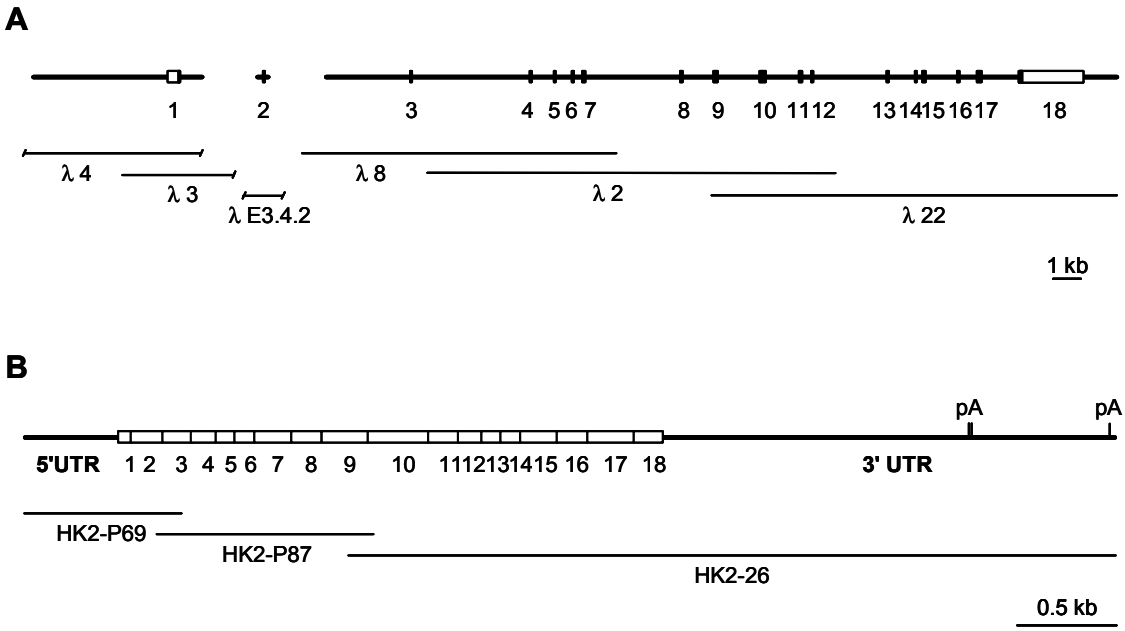



**Figure 4.** Structures of the mouse HKII gene and cDNA. For the genomic structure (A), the sequenced regions are presented as thick lines and the exons as boxes, filled for coding regions and open for untranslated regions. For the organization of mRNA (B), untranslated regions (5' and 3'UTR) are presented as thick lines and the coding region, divided into exons, as open boxes. Putative polyadenylation signals (pA) are denoted above the 3'UTR on the right. For both (A) and (B), the obtained phage clones, with slashes marking off the unknown portions, and cloned RT-PCR fragments (HK2-P69 and HK2-P87) are shown below. Note the considerably different scale in (A) and (B).

Alternative use of one of the two additional putative polyadenylation sites within the 3'UTR of the mouse HKII gene is the likely source for the 4.7 kb minor mRNA. The major transcription initiation site was located at 454 bp upstream of the translation initiation codon in skeletal muscle, heart, adipose tissue and testis by 5'RACE technique. Altogether seven minor sites were identified in all tissues studied. The likely absence of splicing variants involving exon 1 was also demonstrated.

### *5.1.3 cDNA*

Virtually full length mouse HKII cDNA was sequenced (EMBL access number AJ238540) from clones screened from a diaphragm cDNA library, and from RT-PCR products, as depicted (Fig. 4B), and compiled into a pBluescript II SK +/- -vector as pmHK2c. This plasmid contains 420 bp of the 5'UTR, the coding sequence of 2751 bp for 917 amino acids, and the entire 2263 bp 3'UTR.

## 5.2 Generation of the HKII-deficient mouse line (II)

The mouse HKII gene was disrupted in E14 embryonic stem cells by electroporation of a HKII targeting construct containing 9.8 kb isogenic 129/Ola mouse DNA wherein neo gene was inserted into exon 4 (Fig. 3). Out of the obtained 138 neomycin-resistant clones, nine were

determined to be correctly targeted by Southern blotting and PCR (Fig. 5A, B). Three of those (ES4, ES9, and ES10) were aggregated with BALB/c x DBA/2 morulae and placed into the oviducts of pseudopregnant BALB/c x DBA/2 females, resulting in 11 mice with promisingly chimeric coat color. Two of these, derived from clones ES9 and ES10, were both healthy and able to transmit the targeted HKII allele to their offspring, thus facilitating the establishment HKII knock-out mouse lines Ukko1 and Ukko2 (Ukko is short for University of Kuopio knock-out). An additional HKII knock-out line (Ukko3) was also established, but since the founder animal was somewhat retarded in growth and bred poorly, this line was not studied further.

$HKII^{+/-}$ mice of Ukko1 and Ukko2 lines were fertile, produced viable offspring in expected ~1:1 sex ratio, and were of normal weight at birth as well as between weaning (3 weeks) and 27 weeks of age. However, the matings of $HKII^{+/-}$ mice produced wild-type, $HKII^{+/-}$, and $HKII^{-/-}$ offspring (n = 64) in an unexpected 1:2:0 ratio, suggesting prenatal death of $HKII^{-/-}$ mice. Since the initial analyses showed no differences between Ukko1 and Ukko2, further phenotyping was performed only on Ukko1 line. An attempt was also made to breed the male Ukko1 chimera with 129/Ola females (Harlan UK Ltd, Blackthorn, UK) to generate a truly inbred HKII knock-out line, but this proved virtually impossible due to the very poor reproduction capacity of 129/Ola mice.

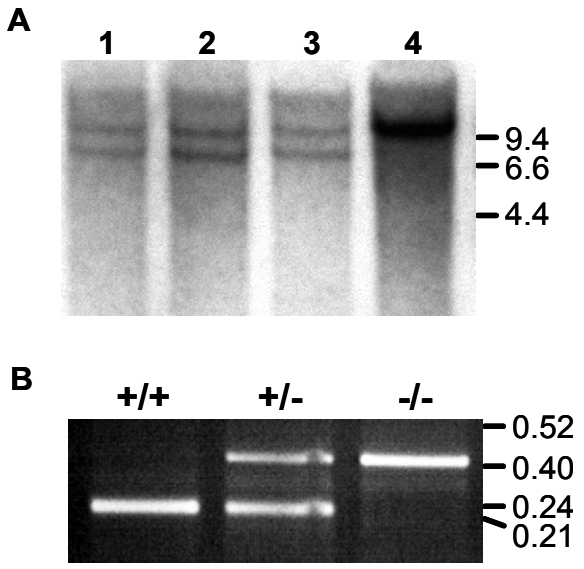



**Figure 5.** (A) Southern analysis of neomycin selected, PCR positive embryonic stem cell clones ES9 and ES10 (lanes 1 and 2), and offspring of Ukko1 mouse line (lane 3, $HKII^{+/-}$; and 4, wild type). Genomic, BamHI digested DNA was hybridized with intronic probe a described in Fig. 3. (B) Exemplary result of the allele discriminating PCR. From the left are shown products for the PCRs of wild-type (+/+) (239 bp), $HKII^{+/-}$ (+/-) (239 and 432 bp), and $HKII^{-/-}$ (-/-) (432 bp) blastocysts. In both (A) and (B), the molecular weight marker as kb is indicated on the right.

## 5.3 Embryonic lethality of $HKII^{-/-}$ mice (II, unpublished)

The death due to the $HKII^{-/-}$ genotype was developmentally staged by PCR-genotyping embryos resulting from $HKII^{+/-}$ x $HKII^{+/-}$ matings. At 3.5, 7.5, and 8.5 days *post coitum* (E3.5, E7.5, and E8.5, respectively), wild-type, $HKII^{+/-}$, and $HKII^{-/-}$ embryos were detected at Mendelian 1:2:1-ratio (**II**, Table 1). However, all E8.5 (**II**, Fig. 2), and 43 % of E7.5 embryos were

macroscopically retarded in growth, whereas all analyzed E3.5 embryos (**II**, Table 1) were viable since they developed normally to blastocysts in an over-night culture of the harvested (at 2.5 days post coitum) morulae.

Further investigations on the developmental effects of $HKII^{-/-}$ genotype were performed using histochemical methods and PCR-genotyping (unpublished). Embryos from $HKII^{+/-}$ x $HKII^{+/-}$ matings were harvested at E6.5 to E8.5, and processed to 8 μm paraffin-embedded sections. Genotyping of each embryo was performed in duplicate on proteinase K treated embryonic material scraped from the sections. As seen in Fig. 6, wild-type embryo at E7.5 displayed all structures typical for this developmental stage, like a visible primitive streak, head fold and notochord, as well as the bulging cephalic mesenchyme flanked by neural ectoderm. However, the two $HKII^{-/-}$ embryos (Fig. 6) were clearly smaller and lacked many of the expected structures, but were surrounded by external membranes that appeared intact. Some internal structures, perhaps neural ectoderm –like, were present, but all in all the embryos appeared growth arrested.

$HKII^{+/-}$ embryos were indistinguishable from wild-type mice of the same sex. In addition, at birth $HKII^{+/-}$ pups had equal body weights to sex-matched wild-type Ukko1 mice (for female wild-type and $HKII^{+/-}$ neonates, 1.35 ± 0.03 and 1.32 ± 0.03 g; for male, 1.36 ± 0.05 and 1.31 ± 0.04 g, respectively; n = 12 to 24 per group) (unpublished).

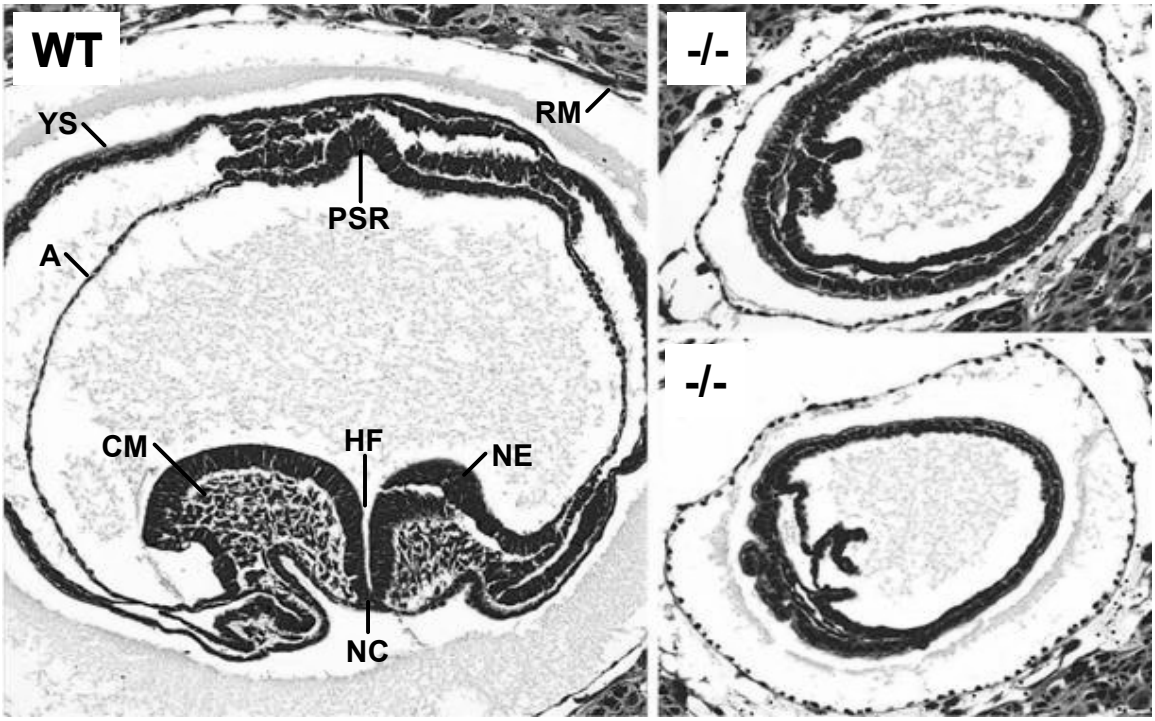



**Figure 6.** Developmental disturbances due to homozygous HKII-deficiency. An embryonic day 7.5 wild-type (WT) embryo on the left displays typical structures at this developmental stage (A, amnion; CM, cephalic mesenchyme, HF, head fold; NC, notochord; NE, neural ectoderm; PSR, primitive streak region; RM, Reichert's membrane; YS, yolk sac). Two $HKII^{-/-}$ embryos (-/-) on the right are retarded in growth and lack most of the normal intraembryonic structures.

## 5.4 Phenotype of HKII$^{+/-}$ mice (II - IV)

Metabolic and other characteristics of the surviving heterozygous HKII knock-out mice were analyzed in different physiological states (basal, high-fat feeding, aging, and exercise) to determine the role of partial HKII deficiency in the control of glucose metabolism at varying levels of insulin resistance and glucose utilization.

### *5.4.1 Basal state (II - IV)*

#### *5.4.1.1 HKII enzyme activity and mRNA expression*

The functional success of HKII knock-out was verified by analyzing HKII gene expression and enzyme activity in perigonadal adipose tissue, the heart, and mixed hind-limb muscle of Ukko1 mice (10-week-old males, n = 4 to 5 per genotype). Northern blot analysis of total RNA demonstrated a reduction in HKII mRNA amount, compared with wild-type mice, in all analyzed HKII$^{+/-}$ mouse tissues (58.9, 50.4 and 62.1 %, respectively) (Fig. 7A). A similar decrease (52.3 %) in HKII$^{+/-}$ quadriceps muscles was demonstrated in another set of Ukko1 mice (6-month-old females, n = 4 to 6 per genotype) by quantitative PCR (**IV**, Table 2). Parallel decrease in HKII enzyme activity was also observed in the same tissues (46.7, 56.0 and 50.5 %, respectively), whereas HKI activity was essentially unchanged (+18.6, -20.8 and +11.7 %, respectively) (Fig. 7B). Also this result was confirmed in another set of 10-week-old female Ukko1 mice (n = 5 to 6 per genotype) (**IV**, Fig. 1).

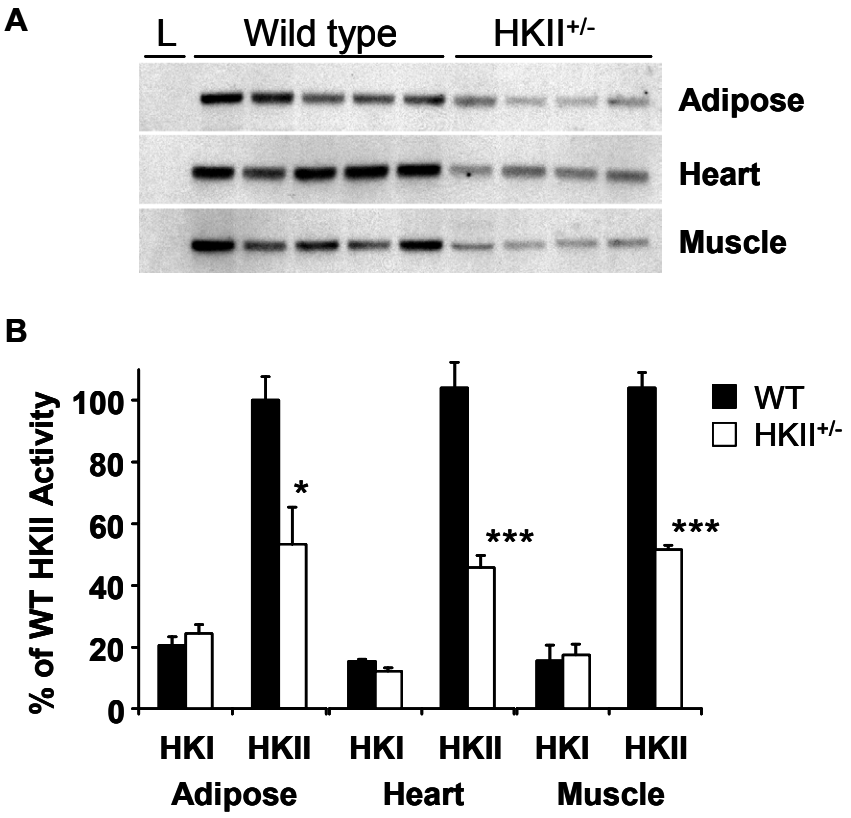



**Figure 7.** Functional verification of the HKII knock-out. Adipose tissue, heart and hind-limb mixed muscle of 5 wild-type and 4 HKII$^{+/-}$ male mice were dissected and used for total RNA extraction and enzyme activity assays. (A) Northern blot analysis of HKII mRNA in the insulin-sensitive tissues of Ukko1 mice. Liver total RNA (L) served as a negative control. The probe encompassed exons 11 to 13 of the mouse HKII coding sequence. (B) HKI and HKII enzyme activities in the insulin-sensitive tissues of Ukko1 mice. Data are presented as mean percent of wild-type HKII activity for each tissue ± SEM. *, $p<0.05$, ***, $p<0.001$.

#### *5.4.1.2 Metabolic phenotype*

As measured at several time-points during maturation from 1 to 22 months of age, sex-matched $HKII^{+/-}$ and wild-type littermates had similar body weight and plasma glucose and insulin values (**II**, Table 2; **III**, Table 1; **IV**, Table 1). In addition, adult $HKII^{+/-}$ mice had circulating levels of cholesterol, triglyceride, and FFA (NEFA) (**II**, text; **III**, Table 1) similar to those of their wild-type counterparts.

Whether anesthetized (**II**, Fig. 5) or non-anesthetized (**IV**, Fig. 3), $HKII^{+/-}$ mice displayed no signs of impaired glucose tolerance or insulin sensitivity. In the GTT of anesthetized $HKII^{+/-}$ males (n = 10 per genotype) (**II**, Fig. 5B), the statistically significant increase in 60 min plasma glucose and insulin values disappeared after correction for the significantly higher body weight of $HKII^{+/-}$ mice present in this particular group of animals. In non-anesthetized $HKII^{+/-}$ females, plasma glucose values tended to be higher in response to *i.p.* glucose load (n = 11 to 13 per genotype), but the difference did not reach statistical significance (**IV**, Fig. 3B).

#### *5.4.1.3 Tissue glucose uptake*

To analyze muscle glucose uptake and metabolite levels in the sedentary state, chronically catheterized, 5 h fasted Ukko1 mice (n = 8 to 11 per genotype) were infused with a 12 μCi bolus of [2-$H^3$]DG, allowed to remain sedentary for a total of 30 minutes, and anesthetized for tissue excision. Sedentary arterial blood samples, collected at regular intervals, showed that there were no differences between wild-type and $HKII^{+/-}$ mice in blood glucose (**III**, Fig. 1A), disappearance of [2-$^3$H]DG from the plasma (**III**, Fig. 2A), or in plasma insulin or FFA (**III**, Table 4) at any time point analyzed. Tissue glucose uptake index $R_g$ (**III**, Table 2) and glycogen content (**III**, Table 3) were also unaltered between the genotypes in all analyzed muscles. Similarly, muscle glucose and G6P content were not different between the genotypes with the exception of G6P in soleus which was reduced by 67 % in $HKII^{+/-}$ mice (**III**, Table 5).

### *5.4.2 High-fat feeding (II)*

Young Ukko1 mice (n = 11 per genotype) were fed high-fat diet between the ages of 4 and 16 weeks. Changes associated with high-fat diet were not seen in plasma glucose or triglyceride levels. Body weight and total cholesterol levels increased, but these were not statistically significant. Plasma insulin levels were markedly higher on high-fat diet than on control diet (5-fold) (**II**, text and Table 2), indicating an induction of insulin resistance. Comparisons of low-fat and high-fat fed insulin sensitivity (**II**, Fig. 6A) and glucose tolerance (**II**, Fig. 6B) supported this. However, $HKII^{+/-}$ genotype did not aggravate insulin resistance over that of wild-type mice.

### 5.4.3 Exercise *(III, IV)*

To analyze muscle glucose uptake and metabolite levels in the sedentary state in Ukko1 mice (n = 11 per genotype), a protocol similar to that in the sedentary state was applied, with the exception that instead of remaining sedentary, mice ran 30 minutes on a treadmill at 0.6 mph (27 cm/s) speed. This moderate-intensity running exercise did not significantly change the level of blood glucose (**III**, Fig. 1B) or plasma FFAs (**III**, Table 4), or the disappearance of [2-$^3$H]DG from the plasma (**III**, Fig. 2B) in either genotype, compared with the sedentary state, nor were there any differences between the genotypes. Plasma insulin levels decreased by ~30 to 40 % due to exercise without significant difference between the genotypes (**III**, Table 4). The heart, and soleus, gastrocnemius and SVL muscles of both wild-type and HKII$^{+/-}$ mice responded to exercise by increasing glucose uptake, but the increase in $R_g$ was less in all HKII$^{+/-}$ tissues than in wild type, albeit statistically significant only in the heart and soleus muscle (Fig. 8A). Exercise resulted in an equal reduction in glucose content of the heart, and soleus and gastrocnemius muscles, but not of SVL, in both genotypes (**III**, Table 5). The tissue levels of G6P remained largely unchanged following exercise, although the soleus G6P content was lower in HKII$^{+/-}$ mice than in wild-type mice (**III**, Table 5). Glycogen breakdown during exercise occurred to the same extent in the heart and SVL in both genotypes, but to greater extent in HKII$^{+/-}$ gastrocnemius (Fig. 8B).

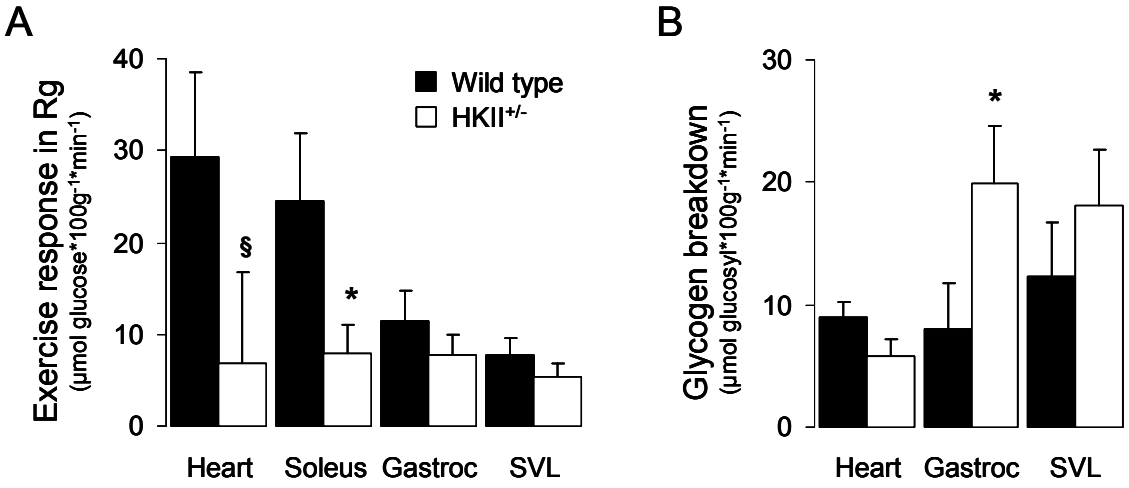



**Figure 8.** Exercise-stimulated response in the muscles of Ukko1 mice on glucose uptake and glycogen breakdown. Fasted, chronically catheterized wild-type and HKII$^{+/-}$ mice (n = 8 to 11 per group) were infused with 12 µCi of [2-$^3$H]-DG during sedentary or exercise (30 min moderate-intensity running) condition. (A) Tissue-specific glucose uptake, Rg, in response to exercise. (B) Glycogen breakdown in response to exercise. For both (A) and (B), Gastroc, gastrocnemius, and SVL, superficial vastus lateralis. Data are presented as means ± SEM. §, $p<0.09$, *, $p<0.05$.

The effect of moderate-intensity exercise on glucose tolerance of adult female Ukko1 mice (n = 10 per genotype) was determined by performing a 90 minute glucose tolerance test while mice were running on a flat-bed treadmill. As demonstrated by significantly higher glucose values of $HKII^{+/-}$ mice at 15, 60 and 90 minutes, and by a significant 14 % increase in the area under the curve (AUC) for glucose (Fig. 9), exercise elicited a mild impairment in glucose tolerance of $HKII^{+/-}$ mice.

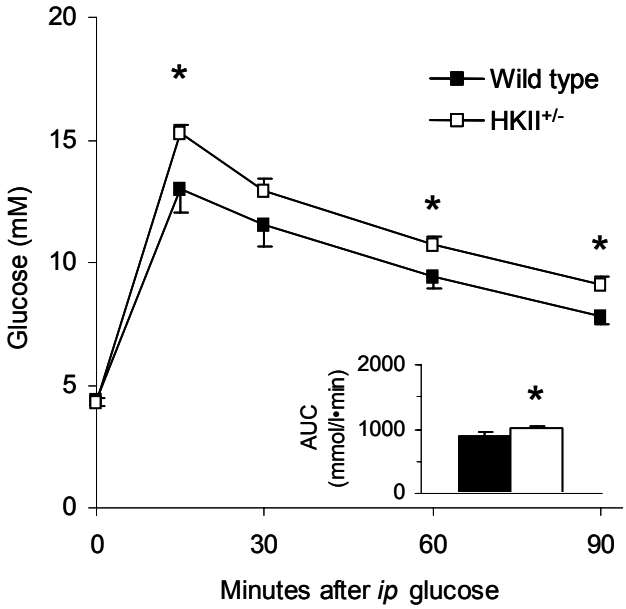



**Figure 9.** Glucose tolerance of Ukko1 mice during exercise. Fasted, wild-type and $HKII^{+/-}$ mice (n = 10 per group) were administered 2 mg/g body weight D-glucose, and placed on a flat-bed treadmill for 90 min moderate-intensity running exercise. The inset depicts the area under the glucose curve (AUC) during the test. Data are presented as means ± SEM. *, $p<0.05$.

### *5.4.4 Aging (IV)*

As analyzed at ~2 years of age, aging did not cause changes associated with $HKII^{+/-}$ genotype in body weight, the weights of individual tissues, or in the levels of plasma metabolites (glucose, lactate, triglycerides or cholesterol) or insulin (n = 8 to 11 per genotype) (**IV**, Table 1). Furthermore, insulin sensitivity and glucose tolerance of aged $HKII^{+/-}$ mice did not differ from those of wild-type mice (Fig. 10A, B). HKII enzyme activity in the soluble fraction of hind-limb muscles in fed 2-year-old (= 104-week-old) $HKII^{+/-}$ mice was decreased by 53 % compared with wild-type mice, while there was no essential change in HKI activity (**IV**, text and Fig. 1). Age-related changes were not observed in HKI or HKII enzyme activity in either genotype when 2-year-old mice were compared with 5 and 10-week-old Ukko1 mice.

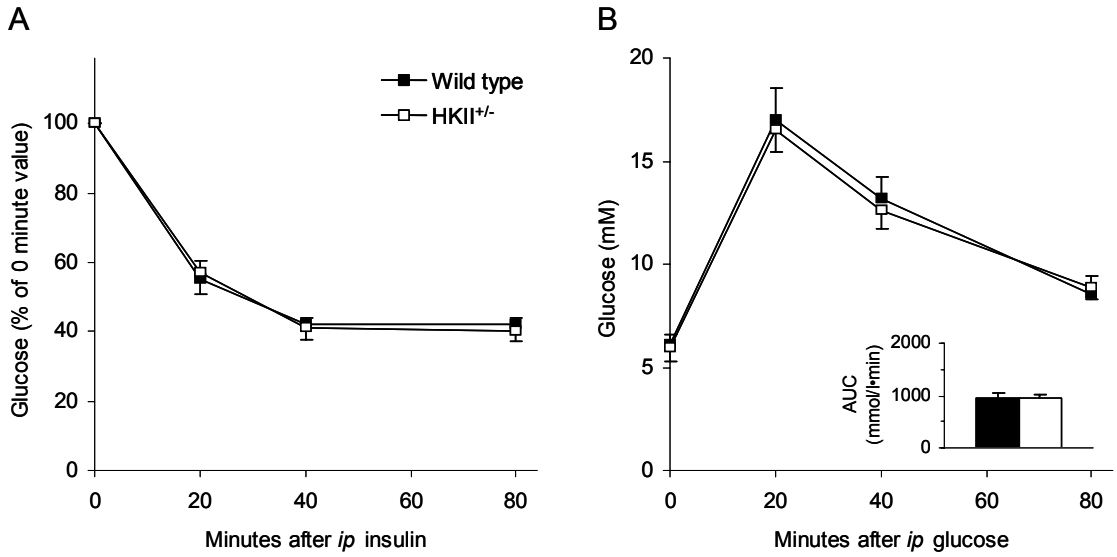



**Figure 10.** Insulin sensitivity and glucose tolerance of aging Ukko1 mice. Wild-type and HKII$^{+/-}$ mice (n = 10 per group) were subjected to (A) *i.p.* insulin tolerance (0.75 mU/g body weight insulin) and (B) glucose tolerance (2 mg/g body weight D-glucose) tests at 2 years of age. The inset depicts the area under the glucose curve (AUC) during the glucose tolerance test. Data are presented as means ± SEM.

## 5.5 Gene expression (IV)

The mRNA expression of several key genes in muscle glucose metabolism was analyzed by quantitative PCR in wild-type and HKII$^{+/-}$ quadriceps muscle (n = 3 to 10 per genotype) both in the sedentary state and after 6 h recovery from 1 h moderate-intensity exercise to investigate possible changes arising from partial HKII-deficiency. The only change associated with HKII$^{+/-}$ genotype in either condition was the 52 to 56 % reduction in HKII mRNA expression (**IV**, Table 2).

# 6 DISCUSSION

Several lines of evidence support the notion that HKII is a good candidate gene for type 2 diabetes. HKII is regulated by insulin and expressed in skeletal muscle and adipose tissue (Katzen, 1966; Katzen and Schimke, 1965; Katzen et al., 1968; Printz et al., 1993; Wilson, 1995; Wilson, 2003). Also, the role of HKII in glucose metabolism is to immediately phosphorylate the transported intracellular glucose to be used as a substrate in glycolysis, and in glycogen and lipid synthesis (Sebastian et al., 2000; Wilson, 1995; Wilson, 2003). In addition, mutations in a member of the HK family, *i.e.* GK, can cause diabetes (Fajans et al., 2001; Froguel et al., 1993). Furthermore, HKII expression is reduced in the tissues of patients with type 2 diabetes despite hyperinsulinemia (Kruszynska et al., 1998; Pendergrass et al., 1998; Vestergaard et al., 1995). For these reasons, it seemed relevant to generate and study HKII knock-out mice to elucidate the role of HKII in glucose metabolism, and perhaps in type 2 diabetes.

## 6.1 Mouse HKII gene and mRNA

The isolated and sequenced fragments of the mouse HKII gene, altogether three genomic regions (Y11666, Y11667 and Y11668) and the virtually complete cDNA (AJ238540), were the largest mammalian HKII sequences of their kind available at the time of publication (**I**), covering most notably 4.8 kb of the promoter, exons 3 to 18 with intervening introns in a large 28.6 kb sequence contig, and the cDNA with almost complete UTRs. The structure of the mouse HKII gene can be considered conventional, as the rather large 2751 bp coding sequence is divided into 18 exons by introns that follow the GT-AG 5' splice donor – 3' splice acceptor consensus. The promoter region contains typical binding sites for basal transcriptional machinery like the TATA and CCAAT-boxes, and the polyadenylation sites in the 3'UTR follow consensus. However, the frequency of simple repeats in the mouse HKII gene is inexplicably high, similarly to the rat HKI gene (White and Wilson, 1997).

Current (**I**) and previous (Malkki, 1999; Malkki et al., 1994; Printz et al., 1993) comparisons of human, rat and mouse HKII genes have shown a high degree (up to 94.9 %) of conservation especially in the coding regions. Similarly, the amino acid sequences of the HKII proteins, deduced from the coding sequences, are very similar between mouse and rat (99.1 %), and mouse and human (94.1 %). A high degree of conservation is also present in the HK family within one species, as the similarity of the deduced mouse HKII and mouse HKI amino acid sequences is 72.1 %. Promoter regions of human, rat and mouse HKII genes are also highly conserved, especially for the first few hundred base pairs of the immediate proximal region, containing important binding sites for factors regulating gene transcription. Furthermore, the location of the

primary transcription initiation site for HKII is identical in the mouse and rat HKII promoters, and very similar to those also in the human promoter.

The primary mouse HKII mRNA species seen in the tissue Northern blot (**I**) is of expected size (~5.5 kb), and it is expressed in the heart, skeletal muscle and adipose tissue, similarly to the rat HKII mRNA (Printz et al., 1993). Minor expression of mouse HKII mRNA was also seen in lung, spleen, ovaries and testes. Expression in the lung has been demonstrated previously also in rat (Printz et al., 1993). However, the relative abundance of the mouse HKII mRNA expression in muscle and lungs differs from that in rat, possibly reflecting species differences or the different methods used (Northern blot vs. RNase protection assay).

A few smaller-than-expected, minor HKII mRNA species are present in the Northern blot, most notably two with sizes of ~4 and ~1.8 kb (**I**). The ~4 kb mouse HKII mRNA apparently results from the use of the two closely located, additional polyadenylation sites present in the 3'UTR of the mouse HKII gene, similarly to human and rat (Printz et al., 1995; Printz et al., 1993). Some or all of the minor HKII mRNA species could be degradation products. Splice variants should not be the reason for the differently sized mRNA species, because such were not detected in this study (**I**), nor have they been reported by others, although other members of the HK-family have alternative promoters (GK) or splicing (HKI) (Magnuson and Shelton, 1989; Mori et al., 1998; Murakami et al., 1999).

The mouse HKII cDNA was cloned into a pBluescript II SK +/- -vector to generate a plasmid pmHK2c (**I**) from which mouse HKII mRNA can be expressed using the viral promoter sequences of the vector. This facilitates its use in expression studies, and by supplementing the mouse HKII cDNA with suitable promoter sequences, also in transfection studies.

## 6.2 Generation of the HKII knock-out mice

Currently, as a result of rapid advances in the public genome projects, whole genomes for human and mouse (Lander et al., 2001; Waterston et al., 2002; Venter et al., 2001), and large continuous chromosomal regions for rat (Twigger et al., 2002), are available, providing complete HKII gene sequences for example within GenBank accessions NT_022184.12 (human), NT_039350 (mouse) and NW_043759 (rat). Had this information been available at the beginning of this study, we still would have obtained the genomic mouse HKII sequences from the 129/SvJ mouse genomic library since the mouse embryonic stem cells available at the time mostly originated from 129/Ola mouse line (**II**), and the use of an isogenic mouse HKII knock-out construct is necessary for the maximal targeting efficiency (Joyner, 1993).

The region of HKII enzyme encoded by exon 4 and onwards contains glucose and ATP binding sites vital for the proper activity (Wilson, 1995). Thus, this region was selected to be included in the HKII targeting construct. In our replacement-type knock-out construct, the positive selection cassette (neo) was inserted into exon 4 to disrupt normal gene function. The construct contained 9.8 kb of isogenic mouse DNA, and was designed to avoid adverse effects of possible exon-skippage (Joyner, 1993), as the exclusion of the disrupted exon 4 from the mature HKII mRNA by the transcription machinery would have resulted in a frame-shift and premature termination codons.

The ES cell clones surviving electroporation with the targeting vector and subsequent neomycin selection were shown by Southern blots and various PCR strategies to contain one correctly targeted HKII allele without additional integrations of the targeting vector (**II**). The importance of thorough verification of correct targeting was demonstrated by one of the original GK knock-out studies (Bali et al., 1995) where an additional integration of the targeting vector at a random site in the mouse genome may have caused inadvertent inactivation of a developmentally important gene, resulting in embryonic lethality which contrasts the other two GK knock-out mouse lines where development to term is not disturbed (Grupe et al., 1995; Postic et al., 1999).

In this study, altogether three HKII knock-out mouse lines (Ukko1-3) were originally generated. To our knowledge, these were the first knock-out mice ever generated in a Finnish laboratory. Whereas $HKII^{+/-}$ mice of Ukko1 and Ukko2 lines grew and bred normally, Ukko3 male founder mouse, derived from the same ES-clone (ES10) as Ukko2, was somewhat retarded and reached fertility as late as at 20 weeks of age. This is in contrast to normal male mice which become fertile as early as at 6 weeks of age. It is not known whether the phenotype of Ukko3 founder mouse resulted from the gene targeting *per se* or was due to natural reasons like random chromosomal defects. Regardless, Ukko3 mouse line was discontinued. After the initial tests showed no differences between Ukko1 and Ukko2 lines, all further studies, discussed below, were performed on Ukko1 mice.

## 6.3 Developmental aspects of HKII-deficiency

Prenatal death of $HKII^{-/-}$ embryos was suspected when $HKII^{+/-}$ x $HKII^{+/-}$ breedings did not produce any $HKII^{-/-}$ offspring. The developmental stage at which $HKII^{-/-}$ embryos die was pinpointed to ~E7.5, meaning that HKII becomes vital to the embryo already before organogenesis, most likely at early gastrulation (**II**). Upon external analysis, $HKII^{-/-}$ embryos were clearly retarded in growth at E8.5, and when analyzed histologically at E7.5 (unpublished),

$HKII^{-/-}$ embryos showed severe retardation in growth and differentiation of embryonic structures typical for the developmental stage.

The death of $HKII^{-/-}$ embryos at an early developmental stage is apparently not compensated by the other three members of the hexokinase family. Although nothing is known about the developmental pattern of HKs during the stages when HKII has a vital role, the lack of compensation is still somewhat surprising since both HKI and HKIII are present in rat skeletal muscle and heart as early as at E14 (Coerver et al., 1998; Griffin et al., 1992). The lack of compensation by GK is not surprising as it evidently does not have an important role in mouse development since complete or tissue specific GK knock-outs do not disturb the *in utero* development of $GK^{-/-}$ mice (Grupe et al., 1995; Postic et al., 1999). $GK^{-/-}$ and $GK^{\beta-/-}$ mice do suffer an early postnatal death, but this is due to disturbed metabolism of ingested nutrients, and not of developmental defects. Another indication of the differential roles for HKII and GK in mouse development is that $HKII^{+/-}$ mice weight as much as wild-type mice at birth (unpublished), whereas $GK^{+/-}$ mice, which later develop mild diabetes, weight less than wild-type mice at birth (Terauchi et al., 2000).

The available data on HKs do not offer information about the specific role of HKII during mouse development, but mice lacking G6P-isomerase (GPI) enzyme (EC 5.3.1.9) provide some insight. GPI-deficient mice, lacking catalysis of the second intracellular step in glycolysis (G6P to fructose-6-phosphate) (Fig. 1), originate from a chemical mutagenesis study (Soares, 1979). Homozygous GPI-mutant mice show striking similarly to $HKII^{-/-}$ embryos (**II**; unpublished) in the timing and histological appearance of the growth arrest (Kelly and West, 1996; Merkle and Pretsch, 1992; West, 1993; West et al., 1990). GPI-mutant mice fail to develop beyond the egg cylinder stage and gastrulation is not completed due to failure to produce a functional mesoderm. Studies on GPI-mutant mice, together with the observation that significant proportion of GPI enzyme in a mouse embryo is oocyte-coded as late as at E4.5 (West et al., 1986), show that glycolysis is vital for the developing embryo at the time of gastrulation (Kelly and West, 1996; West et al., 1990). Additionally, the lack of efficient glycolysis may lead to increased intraembryonic glucose concentration which has been associated with the developmental retardation of preimplantation embryos (Moley et al., 1996). Since the GPI-catalyzed step in glycolysis may be bypassed through pentose phosphate pathway (Fig. 1), it is somewhat surprising that $HKII^{-/-}$ mice survive even as far as the GPI-deficient embryos. One explanation could be the diffusion of metabolites from surrounding tissues as it is the outmost tissues that seem to survive the longest in both $HKII^{-/-}$ (unpublished) and $GPI^{-/-}$ embryos (West et al., 1990). It may also be possible that the oocyte-coded HKs are more abundant or more stable than GPI, or

that HKII has a significant role only after the earliest stages of embryonic development. Whatever the reason for the death of $HKII^{-/-}$ embryos *per se*, or in relation to the death of $GPI^{-/-}$ embryos, it is abundantly clear that HKII has a vital and unique role in early mouse development.

## 6.4 Effects of heterozygous HKII-deficiency

Reliable differentiation of the relative roles of glucose transport and phosphorylation in glucose utilization have proven difficult, although in the basal state, and perhaps also during hyperinsulinemia, transport appears to dominate (Bonadonna et al., 1996; Fink et al., 1992; Furler et al., 1997; Hansen et al., 2000; Ren et al., 1993; Ziel et al., 1988). In contrast, glucose phosphorylation has been suggested to exert primary control in insulin- and especially in exercise-stimulated states (Chang et al., 1996; Furler et al., 1997; Halseth et al., 1998; Halseth et al., 1999; Katz et al., 1991; Kubo and Foley, 1986; Rossetti and Giaccari, 1990), although also for exercise there is evidence to the contrary (Hansen et al., 1995). In type 2 diabetes, nuclear magnetic resonance (NMR) studies (Petersen and Shulman, 2002) have shown that glucose transport is the rate-limiting step for insulin-mediated glucose uptake in skeletal muscle, but again, contrasting results have been obtained, favoring the major role of glucose phosphorylation (Saccomani et al., 1996). In studies discussed here (**II**-**IV**), we investigated whether partial HKII deficiency has a significant effect on glucose metabolism when the rate thereof was varied within the physiological range.

### *6.4.1 Basal state*

Gene-dose dependent, ~50 % decrease in HKII mRNA expression and enzyme activity in the HKII expressing tissues of $HKII^{+/-}$ mice (**II**, **IV**) clearly demonstrates the functional success of HKII knock-out, in addition to the lethality of homozygous HKII-deficiency (**II**). The lack of compensatory increase in HKI enzyme activity, or in the remaining HKII activity, in $HKII^{+/-}$ mice might suggest that the metabolic consequences of heterozygous HKII-deficiency are too mild to elicit such a compensation. However, this is not supported by GK and GLUT4 knock-out studies, since in $GK^{+/-}$ mice, a similar ~50 % reduction in GK activity persists despite mild diabetes (Bali et al., 1995; Grupe et al., 1995; Postic et al., 1999), and in $GLUT4^{+/-}$ mice, a similar reduction in the amount of muscle GLUT4 protein persists despite insulin resistance (Rossetti et al., 1997b). It should be noted that the applicability of rodent HK-data to human physiology may be limited since in human muscle, HKII accounts for only 25 to 50 % of total HK activity (Kruszynska et al., 1998; Ritov and Kelley, 2001; Vestergaard et al., 1995; Vogt et al., 1998), whereas in rat (Grossbard and Schimke, 1966; Lawrence and Trayer, 1985) and mouse (**II, IV**) (Chang et al., 1996) muscle, HKII predominates (~70 to 90 %). The difference in muscle HKI / HKII ratio

between humans and rodents may reflect better protection of human muscle bioenergetics during exercise (Ritov and Kelley, 2001).

In striking contrast to the heterozygous GK and GLUT4 knock-out mice, $HKII^{+/-}$ mice did not show changes in physiological and metabolic indices analyzed in the basal state (**II** - **IV**), although there was a trend towards impaired glucose tolerance in non-anesthetized $HKII^{+/-}$ mice. In the basal state, also muscle metabolites and glucose uptake were unaltered in $HKII^{+/-}$ mice (**III**). Thus, in agreement with data from HKII overexpressing mice (Chang et al., 1996; Halseth et al., 1999; Lombardi et al., 1997), glucose phosphorylation is not rate-limiting for mouse glucose metabolism in the basal state, not even when there is a 50 % lack in the insulin-stimulated glucose phosphorylation capacity.

### *6.4.2 High-fat feeding*

High fat intake results in insulin resistance, impaired glucose tolerance (Kim et al., 2000; Kraegen et al., 1991; Storlien et al., 1986; Surwit et al., 1988; Susini and Lavau, 1978; Vessby, 2000), and reduced rates of insulin-stimulated glucose uptake in skeletal muscle and adipose tissue via GLUT4 repression (Sevilla et al., 1997; Zierath et al., 1997). In addition, although high-fat diet prevents the normal post-weaning increase in muscle HKII mRNA in rats (Postic et al., 1994), high-fat diet does not affect muscle HKII enzyme activity in adult rodents (Baxter and Schofield, 1980; Kim et al., 2000; Zierath et al., 1997). Thus, glucose phosphorylation could become rate-limiting for glucose metabolism during a high-fat diet to such an extent that partial HKII deficiency would exacerbate the diet-induced metabolic disturbances. However, although chronic high-fat feeding clearly induced insulin resistance in Ukko1 mice, as demonstrated by impaired glucose tolerance and increased fasting insulin levels, there was no difference between these or any other measured indices between wild-type and $HKII^{+/-}$ mice (**II**). Although we failed to measure the effect of high-fat diet on HKII enzyme activity and mRNA to verify the continued 50 % HKII deficiency also after the diet, it may be concluded that glucose transport retains primary control of glucose metabolism in response to hyperglycemia also during a high-fat diet even in mice with heterozygous genetic HKII-deficiency (**II**).

### *6.4.3 Exercise*

Exercise increases muscle glucose utilization by augmenting glucose delivery via increased muscle perfusion, and by enhancing glucose transport via increased GLUT4 translocation to the plasma membrane (Bryant et al., 2002; Richter et al., 2001; Wasserman and Halseth, 1998). Although exercise also increases muscle phosphorylation capacity by augmenting HKII gene expression and enzyme activity (Hildebrandt et al., 2003; Koval et al., 1998; Nordsborg et al.,

2003; O'Doherty et al., 1996; O'Doherty et al., 1994; Pilegaard et al., 2000), glucose phosphorylation still becomes rate-limiting in the control of muscle glucose utilization (Fueger et al., 2003; Halseth et al., 1998; Halseth et al., 1999). Furthermore, overexpression of HKII in mouse skeletal muscle increases exercise-stimulated glucose uptake, suggesting that glucose phosphorylation by HKII controls muscle glucose utilization during exercise (Fueger et al., 2003; Halseth et al., 1999). Based on these data, we hypothesized that a decrease in HKII activity is likely to result in an impaired muscle glucose uptake during exercise (**III**), which might also lead to impaired glucose tolerance (**IV**).

The exercise-stimulated increase in glucose uptake was impaired in oxidative $HKII^{+/-}$ muscles (the heart and soleus). In glycolytic $HKII^{+/-}$ muscles (gastrocnemius and SVL), the impaired uptake of external glucose was apparently compensated by increased glycogen breakdown, providing more glucose substrate from an intracellular pool (**III**). It is noteworthy that the correlation seen in wild-type mice between the oxidative capacity of a muscle (Delp and Duan, 1996; Grange et al., 2001; Harrison et al., 2002; Hughes et al., 1999) and the magnitude to which muscle glucose uptake was stimulated by exercise was not present in $HKII^{+/-}$ mice (**III**). In the fasted state, exercise does not seem to disturb glucose metabolism of $HKII^{+/-}$ mice, as demonstrated by the lack of difference between wild-type and $HKII^{+/-}$ mice in blood glucose and plasma [2-$H^3$]DG values throughout the exercise period (**III**). These results have opened several lines to further studies. For example, it is of interest to determinate the mechanism by which the glycogen stores of the heart are spared despite marked impairment in exercise-stimulated glucose uptake. In addition, it would be worthwhile to investigate whether the glycolytic muscles could be stimulated to such a level, in respect to their glycogen content, that the apparent impairment in glucose uptake would become directly measurable (**III**).

Whole-body glucose utilization on Ukko1 mice in response to a combination of exercise and hyperglycemia was investigated by performing a 90-minute GTT during moderate-intensity exercise (**IV**). In these conditions, $HKII^{+/-}$ mice became mildly glucose intolerant, showing that even a moderate (50 %) decrease in insulin-stimulated glucose phosphorylation capacity in muscle and adipose tissue is enough to disturb whole-body glucose metabolism (**IV**).

Taken together, our observations on the effects of exercise on muscle glucose uptake and glucose tolerance in $HKII^{+/-}$ mice (**III**, **IV**) show that the controlling step of glucose metabolism, both at the level of muscle as well as at the level of the whole body, can shift from the usual transport step to phosphorylation. However, while the glucose utilization system is flexible in shifting the controlling step as needed, it can also be disturbed even by heterozygous mutations in enzymes catalyzing the respective rate-limiting steps. Thus, our data is in complete agreement with what

has been previously shown for heterozygous GLUT4 and GK knock-out mice which have disturbed glucose metabolism and at least mild diabetes (Bali et al., 1995; Grupe et al., 1995; Postic et al., 1999; Rossetti et al., 1997b; Stenbit et al., 1997).

### 6.4.4 Aging

Aging is associated with increased insulin resistance and impaired glucose tolerance (Davidson, 1978; Goodman et al., 1983; Narimiya et al., 1984; Petersen et al., 2003; Reaven et al., 1989), and reduced levels of GLUT4 (Lin et al., 1991; Sevilla et al., 1997). In addition, aging has been shown to result in decreased metabolite concentrations and mitochondrial and glycolytic enzyme activities in skeletal muscle, including HK (Cartee, 1994; Pastoris et al., 2000; Stump et al., 1997). Thus, we investigated parameters of glucose metabolism in ~2-year-old female Ukko1 mice to define whether age-related reduction in glycolytic capacity causes further disturbances in glucose metabolism (**IV**). However, HKI and HKII enzyme activities in hind-limb mixed muscle of 2-year-old Ukko1 mice were no different from those of adolescent or young adult Ukko1 mice. Furthermore, aging did not alter the gene-dose dependent reduction of HKII activity in $HKII^{+/-}$ muscles. Thus, these data suggest that in mouse mixed hind-limb muscle, aging does not result in reduced glucose phosphorylation capacity. Aging in skeletal muscle is associated with reduced levels of cell surface GLUT4, at least in rats (Lin et al., 1991; Sevilla et al., 1997). In contrast, HKI and HKII activities are well-preserved upon aging, as shown here for the mouse. It is therefore not likely that glucose phosphorylation is rate-limiting for glucose metabolism in aging mice. Thus, it is not surprising that differences in glucose tolerance or insulin sensitivity were not observed between 2-year-old wild type and $HKII^{+/-}$ mice. In fact, our results seem to suggest that aging *per se* does not cause disturbances in insulin-sensitive glucose metabolism in these mice. Nevertheless, these results show that aging does not lead to impaired glucose metabolism in partial HKII deficiency (**IV**).

### 6.4.5 Gene expression

The mRNA expression of several key glycolytic genes, including HKII, GLUT4, GPI, phosphofructokinase, and glycogen synthase, as well as some important insulin signalling molecules like IRS1 and PPARγ, was studied both in the basal state and after exercise in Ukko1 mouse quadriceps muscles (**IV**). In both states, HKII was decreased as expected, but significant changes in the expression of other genes were not observed. Although compensatory changes in other genes not investigated can not be ruled out, this result may support the contention that the physiological consequences of $HKII^{+/-}$ genotype seen during exercise are too mild to cause changes in the regulation of gene expression. However, this would be partly in contrast to the

data from GLUT4-null mice where compensation by an as yet unidentified mechanism occurs (Katz et al., 1995; Ryder et al., 1999a).

## 6.5 Concluding remarks

In this study, new data were provided for the organization and expression of mouse HKII gene. When the mouse HKII gene was knocked out, a vital and unique role for HKII in the early stages of mouse development was discovered. The phenotype of the non-lethal heterozygous HKII inactivation in mouse confirmed that glucose phosphorylation is not likely to be rate-limiting in glucose metabolism in the basal state, or upon high-fat feeding or aging. However, glucose phosphorylation by HKII was shown to be rate-limiting in both muscle glucose uptake and glucose tolerance during moderate-intensity exercise. Compensatory changes in the expression of key glycolytic and insulin signalling genes were not observed.

From the clinical point of view, the consensus from studies on animal models with altered glucose metabolism in muscle presented both here and elsewhere seems to be that enhancing glucose transport rather than phosphorylation by pharmacological or other means might be beneficial in the treatment of hyperglycemia or insulin resistance. Nevertheless, the data presented in this study provides new avenues for further study of $HKII^{+/-}$ mice on the effects of defective glucose phosphorylation especially during exercise. In addition, studies on the effects of complete loss of HKII activity in adult mice will become feasible if the embryonic lethality of $HKII^{-/-}$ mice could be bypassed by conditional knock-out technology.

# 7 SUMMARY

The principal aim of this study was to elucidate the role of HKII in mouse development and glucose metabolism using a HKII knock-out mouse model. As means to this end, secondary aims were to clone and characterize the mouse HKII gene, and construct the HKII targeting vector. The following results were obtained.

I Mouse HKII gene and full length cDNA were cloned, sequenced and characterized, and shown to be closely related to previously characterized rat and human sequences. The tissue distribution of the mouse HKII mRNA expression was determined, and found similar to what is known for rat and human. Mouse HKII gene promoter organization, and mRNA transcription initiation and termination sites were also determined.

II A mouse HKII gene targeting vector was constructed and used to inactivate a HKII allele in mouse embryonic stem cells. Of the three subsequently obtained HKII knock-out mouse lines, one (Ukko1) was characterized further. The vital and unique role for HKII in mouse development was discovered when homozygous HKII knock-out embryos died at embryonic day ~7 to 8, suggesting a defect in gastrulation. In contrast, heterozygous HKII knock-out mice were viable and had an approximately 50 % decrease in HKII mRNA and enzyme activity in the heart, skeletal muscle and adipose tissue, but their fasting or fed levels of circulating glucose and other metabolites and hormones were not altered. Furthermore, $HKII^{+/-}$ mice were as glucose tolerant and insulin sensitive as wild-type mice in the basal state, as well as after diet-induced obesity and insulin resistance. This confirmed that glucose phosphorylation by HKII is not rate-limiting for glucose utilization in mice either in the basal state or after high-fat feeding.

III The effect of moderate-intensity running exercise on the glucose metabolism of wild-type and $HKII^{+/-}$ mice was investigated by analyzing their circulating metabolite and insulin levels, muscle metabolite content, and the amount of tissue glucose uptake. In general, wild-type and $HKII^{+/-}$ mice did not differ from each other in the sedentary state. However, exercise elicited a significantly smaller increase in $HKII^{+/-}$ glucose uptake index, $R_g$, compared with wild type, in oxidative muscles (heart and soleus) while there was a similar trend in glycolytic muscles (gastrocnemius and SVL). The lack of a significant difference in $R_g$ in glycolytic $HKII^{+/-}$ muscles may have resulted from their apparent ability to compensate by increased exercise-stimulated glycogen breakdown. Thus, muscle glucose uptake is impaired by a reduction in HKII activity

during exercise-stimulated high glucose flux. This impairment critically depends on the muscle glucose metabolic rate and correlates with tissue oxidative capacity.

IV The metabolic phenotype of $HKII^{+/-}$ mice was investigated further by analyzing aging mice, and adult mice during exercise. Adult mice were also analyzed in the basal state without anesthesia. Aging did not bring about alterations in $HKII^{+/-}$ mice in the measured metabolic parameters. Neither did the omission of anesthesia. With the obvious exception of HKII, the mRNA expression of several genes for key glycolytic enzymes and insulin signalling molecules was not altered, either in the basal state or after exercise. However, it was shown that glucose tolerance of adult $HKII^{+/-}$ mice is impaired during moderate-intensity running exercise, suggesting that glucose phosphorylation by HKII is rate limiting in whole-body glucose utilization when both demand and supply of glucose are high.

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