Both isoforms of ketohexokinase are dispensable for normal growth and development


1 Leeds Institute of Molecular Medicine, University of Leeds, St James’s University Hospital, and 2 School of Chemistry, University of Leeds, Leeds, United Kingdom

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Diggle CP, Shires M, McRae C, Crellin D, Fisher J, Carr IM, Markham AF, Hayward BE, Asipu A, Bonthron DT. Both isoforms of ketohexokinase are dispensable for normal growth and development. Physiol Genomics 42A: 235–243, 2010. First published September 14, 2010; doi:10.1152/physiolgenomics.00128.2010.—Dietary fructose intake has dramatically increased over recent decades and is implicated in the high rates of obesity, hypertension, and type 2 diabetes (metabolic syndrome) in Western societies. The molecular determinants of this epidemiologic correlation are incompletely defined, but high-flux fructose catabolism initiated by ketohexokinase (Khk, fructokinase) is believed to be important. The Khk gene encodes two enzyme isoforms with distinctive substrate preferences, the independent physiological roles of which are unclear. To investigate this question, and for testing the importance of Khk in metabolic syndrome, isoform-selective genetic lesions would be valuable. Two deficiency alleles of the mouse Khk gene were designed. The first, Khkα3 (α), uses targeted “knock-in” of a premature termination codon to induce a selective deficiency of the minor Khk-A isoform, preserving the major Khk-C isoform. The second, the Khkβ3 allele, ablates both isoforms. Mice carrying each of these Khk-deficiency alleles were generated and validated at the DNA, RNA, and protein levels. Comparison between normal and knockout animals confirmed the specificity of the genetic lesions and allowed accurate analysis of the cellular distribution of Khk within tissues such as gut and liver. Both Khkα3/α3 and Khkβ3/β3 homozygous mice were healthy and fertile and displayed minimal biochemical abnormalities under basal dietary conditions. These studies are the first demonstration that neither Khk isoform is required for normal growth and development. The new mouse models will allow direct testing of various hypotheses concerning the role of this enzyme in metabolic syndrome in humans and the value of Khk as a pharmacological target.

IN NATURE, dietary fructose (in the form of sucrose) is largely derived from fruit and vegetables, but in Western societies fructose intake has risen dramatically over the last few decades, partly because of the use of high-fructose corn syrup as a commercial sweetener. Much evidence (both epidemiologic and from animal studies) indicates that high dietary fructose predisposes to obesity, metabolic syndrome, hypertension, hyperuricemia, diabetes, and renal disease (5, 15, 21, 35–37).

High-fructose diets can increase the levels of triglycerides, uric acid, and insulin, themselves risk factors for the development of obesity and other diseases (20, 26). The peculiar effects of fructose reflect the relationship between the separate catabolic pathways for fructose and glucose. Fructose catabolism commences with its phosphorylation, predominantly to fructose-1-phosphate (F1P) by ketohexokinase (Khk). Expressed at highest level in the liver (1), the perceived primary role of Khk is in the clearing of postprandial fructose from the portal bloodstream. Khk-derived F1P is subsequently metabolized by aldolase B and triokinase to dihydroxyacetone phosphate and glyceraldehyde-3-phosphate, which enter the latter stages of glycolysis. Fructose catabolism initiated by Khk therefore bypasses the major glycolytic checkpoint at phosphofructokinase, resulting in increased lipogenesis compared with glucose. F1P itself also regulates hepatic glucose metabolism, since its binding to the glucokinase regulatory protein (GKRP) allows glucokinase to translocate from nucleus to cytoplasm (8, 38). This increases glucose conversion to glucose-6-phosphate.

Fructose-induced hyperuricemia results from rapid hepatic ATP consumption, leading to elevated AMP deaminase activity and increased uric acid production (22, 34). The mechanism by which fructose induces hyperinsulinemia is less well understood (26).

The human and mouse Khk genes comprise nine exons, two of which, 3a and 3c, are adjacent (reflecting an ancestral duplication) and are subject to mutually exclusive splicing into mRNAs that encode Khk-A or Khk-C isoforms, respectively (16). The isoforms have comparable maximum catalytic activities, but only Khk-C appears adapted for dietary fructose clearance (km = 0.8 mM), while Khk-A has a 10-fold higher km (3).

It is the Khk-C isoform that is expressed in liver, kidney, and small intestine. Humans deficient in HKH-C manifest the rare benign disorder essential fructosuria (6, 7, 31), in which 10–15% of a dietary fructose load is lost in the urine. In contrast, the Khk-A isoform is expressed at low level in a wide range of tissues, and its physiological substrate remains unknown (36).

To address the latter question, and to generate animals useful for testing the importance of Khk in the diet-related metabolic pathologies referred to above, two mouse knockout models have been generated. One strain is selectively deficient in peripheral Khk-A (the Khkα3/α3 mouse), while the second strain lacks activity of both Khk-A and Khk-C (Khkβ3/β3). Here, the generation and initial molecular and biochemical characterization of these animals are described.

MATERIALS AND METHODS

Generation of mouse models. To construct the Khk gene targeting vectors, Khk-containing genomic clones were isolated from mouse strain 129S6/SvEvTac by screening the RPCI-21 PAC library [Medical Research Council (MRC) Geneservice]. To generate a Khk-A-
selective genetic lesion (targeting vector 1), an in-frame stop codon was engineered into exon 3a. For positive selection, a floxed neo cassette from PGKneoB- lox (39) was inserted 5' to exon 3a. For negative selection, a PGK-DTA cassette (kindly provided by P. Soriano, Fred Hutchinson Cancer Research Center, Seattle, WA) was inserted 3' to Khk. Both selectable markers were in opposite transcriptional orientation to Khk. To engineer targeting vector 2 (for generating a Khk null allele), a region between exons 3c and 7 of targeting vector 1 was deleted. Detailed methods are provided in the Supplemental Material for this article.1

The targeting vectors were linearized with NotI and electroporated into R1 embryonic stem (ES) cells (MRC Mary Lyon Centre, Harwell, UK). After G418 selection, correctly targeted recombinant clones containing the Khk3a or the Khk2a allele were identified by long PCR and Southern blotting. In addition, sequencing confirmed the presence of the stop codon in exon 3a of the recombined locus. Further details are in the Supplemental Material.

Selected ES clones (8H and 9E for Khk3a and 1A and 5C for Khk2a) were injected into C57BL/6J blastocysts at the MRC Mary Lyon Centre. Chimeras were then mated with C57BL/6 animals, and PCR was used to identify the Khk genotype of offspring. More detailed methods are in the Supplemental Material.

To minimize possible interference with splicing by the neo cassette, animals carrying a correctly targeted Khk3a allele were crossed with a homozygous Cre recombinase-expressing strain, FVB/N-Tg (Actb-cre)2Mrt/J, to delete the floxed neo. Approximately 100 bp of vector-derived sequence outside of the loxP site remained inserted at the genomic EcoRV site after Cre excision. This allowed discrimination between the unrecombined wild-type (428 bp) and Cre-excised Khk3a (~530 bp) alleles with the EcoRV-flanking primers (Supplemental Table S1). To eliminate the Cre transgene from the strains, Cre-excised Khk3a heterozygotes were further crossed with C57BL/6 and progeny were screened by PCR to identify Khk3a heterozygotes without the Cre transgene (Supplemental Table S1).

Sample collection. A project license approving this work was granted by the Home Office (United Kingdom) as required by the Animals (Scientific Procedures) Act 1986. Animals were maintained on a standard rat and mouse diet (BK001E, B&K Universal, Hull, UK). Animals were killed with carbon dioxide and cervical dislocation on a standard rat and mouse diet (BK001E, B&K Universal, Hull, UK). After G418 selection, correctly targeted recombinant clones into R1 embryonic stem (ES) cells (MRC Mary Lyon Centre, Harwell, UK). After G418 selection, correctly targeted recombinant clones containing the Khk3a or the Khk2a allele were identified by long PCR and Southern blotting. In addition, sequencing confirmed the presence of the stop codon in exon 3a of the recombined locus. Further details are in the Supplemental Material.

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Tissue metabolite analysis. All animals were ~12 wk old. From the Khk3a strains, tissues were collected from eight wild-type, eight heterozygous, and eight homozygous females. From the Khk2a/3a strain, tissues were collected from six wild-type and six Khk2a/3a animals, which had been fasted for 8 h.

Tissue extracts were performed, and glucose and fructose concentrations were measured with a UV-method assay kit (Boehringer Mannheim/R-Biopharm, Glasgow, UK). Sorbitol was measured with a colorimetric method assay kit (Boehringer Mannheim/R-Biopharm) as described previously (17). For fructose determination in kidney, glucose was first removed with glucose oxidase, essentially as described previously (29). Statistical analysis was by one-way ANOVA for the Khk3a animals and by t-test or Mann-Whitney test for the Khk2a animals.

Plasma metabolite analysis. All animals were ~12 wk old. Measurements of glucose, creatinine, cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, and uric acid were purchased from the Clinical Pathology Laboratory, MRC Mary Lyon Centre. Insulin levels were assessed with an ultrasensitive mouse insulin ELISA kit (Crystal Chem, Downers Grove, IL). Samples were collected from eight each of Khk3a/3a homozygotes and wild-type and heterozygous littersmates and from five each of homozygous Khk2a/3a animals and wild-type littersmates, fasted for 8 h. Statistical analysis was by two-way ANOVA, two-tailed t-test, or Mann-Whitney test.

Proton nuclear magnetic resonance (NMR) spin-echo spectra (25) were collected by a Varian Unity Inova Spectrometer with an operating frequency of 500 MHz (full data collection details are provided in the Supplemental Material). Plasma from nonfasted animals was added to deuterium oxide. Resultant spectra were data reduced by binning and analyzed by principal component analysis (PCA) with SIMCA-P+ 12.0.1 software (Umetrics, Umeå, Sweden). Significance was tested with SPSS 15.0 software (SPSS, Chicago, IL). After tests of normality, the Mann-Whitney U-test was performed to compare the mean values of the integrals from influential regions of the NMR spectra. Resulting P values were adjusted for multiple comparisons using false discovery rate with R 2.7.0 software (R Foundation for Statistical Computing, Vienna, Austria).

1 Supplemental Material for this article is available online at the Journal website.
RESULTS

**Generation of Khk knockout mice.** A standard positive/negative selection method (using G418 resistance and diphtheria toxin markers, respectively) was used to target the Khk locus in strain 129 ES cells by homologous recombination (Fig. 1). The Khk-A-specific allele (Khk<sup>3a</sup>) was generated by introducing a stop codon into exon 3a (CGA→TGA; Arg<sup>84</sup>→stop). The Khk<sup>Δ</sup> allele, ablating both Khk isoforms, was generated by deleting a fragment including sequence from Khk exons 4–7 (Fig. 1D).

Approximately one-third of 200 ES cell clones selected after transfection with the Khk<sup>3a</sup> construct were correctly targeted at the Khk locus. A similar percentage was obtained with the Khk<sup>Δ</sup> construct. Southern blotting using various combinations of the BLASTP tool from NCBI, or other similar protein BLAST tools, could help identify the specific interaction of Khk with other proteins. The targeting vectors (shown in more detail in Supplemental Figs. S1–S3) encompassed a 15.5-kb genomic segment between upstream NotI and downstream NdeI sites. In A, dotted lines indicate the normal mutually exclusive splicing patterns of exons 3a and 3c. The initial targeting event (B) introduces an in-frame stop codon (TGA) into exon 3a. To minimize interference with Khk transcription and splicing, the Neo cassette was subsequently excised from the germ line with Cre recombinase (C). The Khk<sup>Δ</sup> null allele (D) was generated similarly, with a derivative targeting vector from which a 3.6-kb ApaI fragment (exons 4–7; sites marked as “<” and “>” in A) had been deleted. In this case, the Neo cassette was left in situ. E: Southern blot of BssSI-digested DNA (see A and B) to identify correctly targeted heterozygous embryonic stem (ES) cell clones (lanes 1–6). Lane 7, normal mouse DNA; M, molecular weight marker. F: After crossing with a Cre recombinase expressor, the PCR indicated in C is used to identify pups heterozygous for the final targeted allele, by virtue of the -100-bp insertion at the EcoRV site.
of restriction enzymes and probes confirmed that several clones had a single correct homologous recombination event at the Khk locus (Fig. 1E). Two ES cell clones per targeting construct were selected for injection into C57BL/6J blastocysts.

The resulting chimeras were crossed with C57BL/6J, to establish germ line transmission. The Khk3a animals were next crossed with Cre recombinase-expressing mice to remove the neomycin cassette, correct excision of which was confirmed by PCR (Fig. 1F). These animals were further crossed with C57BL/6 mice. Animals that were heterozygous for the Khk3a allele but had not inherited the Cre transgene were used in subsequent crosses and experiments.

Characterization of knockout animals. Matings between homozygotes (either Khk3a/3a or Khk3a/3b) resulted in normal-sized healthy litters, thus establishing that loss of Khk does not affect fertility or normal development to term.

Khk transcript analysis. Real-time PCR was used to analyze the Khk-A transcript levels in lung tissue, known to express Khk-A but not Khk-C (13, 16), of wild-type, heterozygous Khk3a/+, and Khk3a/3a mice (Fig. 2A). In Khk3a/3a animals, Khk-A mRNA is reduced to ~10% of normal (P < 0.05), presumably as a result of non-sense-mediated decay. (The residual Khk-A mRNA, of course, will encode a truncated protein.) In heterozygotes, the Khk-A mRNA level was approximately half that of wild-type animals (P < 0.05). The same significant expression level differences were seen in two additional Khk-A-expressing tissues, brain and spleen (data not shown). Khk-C mRNA levels were also analyzed, to determine whether the reduction in Khk-A transcript was compensated for by a switch to Khk-C; no such effect was observed (data not shown).

In the Khk3a/3a animals, analysis of normally Khk-C-expressing tissue (liver) revealed, as expected, virtually no Khk-C transcript (Fig. 2B). Heterozygous animals again had approximately half the level of transcript of wild-type animals. These differences were statistically significant (P < 0.05). Similar results were obtained in another Khk-C-expressing tissue, kidney (data not shown).

Immunodetection of Khk protein. Immunodetection of the low levels of Khk in Khk-A-expressing tissues has been shown to be difficult; in addition to the 30-kDa Khk-A protein, many cross-reacting bands appear on Western blots, some of higher
signal intensity than the Khk signal (13). To alleviate this, the thermostability of the Khk-A protein was exploited (3) by heating the protein extracts before electrophoresis to precipitate many cross-reacting proteins. This permitted the detection of a faint 30-kDa band in spleen (Fig. 2C) and heart (not shown) of wild-type and heterozygous animals, which was absent from the corresponding Khk<sup>3a/3a</sup> tissues.

Western blot analysis of a highly expressing Khk-C tissue (liver), in contrast, was much easier to interpret. A prominent 30-kDa immunoreactive band was clearly missing from extracts of Khk<sup>3a/3a</sup> liver (<i>P < 0.05</i>) (Fig. 2D). Quantification of the Khk band intensity in relation to β-actin further indicated approximately half the level of Khk in the heterozygous liver samples compared with wild type (<i>P < 0.05</i>). Similar results were also seen on Western blotting of kidney extracts from wild-type and Khk<sup>3a/3a</sup> animals (not shown).

Khk enzyme activity. A coupled enzyme assay was employed, which measures Khk activity indirectly, through the consumption of ATP (2). As expected, in crude liver extracts from Khk<sup>3a/3a</sup> mice enzyme activity was undetectable (Fig. 2E). Heterozygotes manifested 50% of wild-type activity; the differences between all three groups were statistically significant (<i>P < 0.05</i>). In Khk-A-expressing tissues (such as lung), this assay does not allow Khk activity to be distinguished from background ATP consumption by other enzymes. Consequently, no differences between extracts of lung from Khk<sup>3a/3a</sup> and wild-type mice were demonstrable (not shown).

Immunohistochemistry. Previously, significant nonspecific reactivity was found when using a chicken anti-KHK antibody (see MATERIALS AND METHODS) for immunohistochemical analysis of the pancreas, a tissue expressing low levels of both Khk isoforms (13). In tissues expressing a low level of Khk-A (brain, spleen, lung, and heart), again no differences were discernible between wild-type, heterozygous, and homozygous Khk<sup>3a/3a</sup> animals, because of the high level of nonspecific antibody binding (Supplemental Fig. S6). In contrast, in the high-Khk-C-expressing tissues (kidney, liver, small intestine) specific Khk immunolocalization is readily discernable by comparison of normal and Khk<sup>3a/3a</sup> animals. This previously allowed the demonstration of proximal tubule localization of Khk in the kidney (13). Both here and in the liver (Fig. 3, A–C), there is both cytoplasmic and intense nuclear staining. Heterozygous animals did not display noticeable differences in staining from wild-type littermates (Fig. 3, A–C).

Small intestine is another high-Khk-C-expressing tissue. Although there was some nonspecific antibody reactivity in the subepithelial interstitium and luminal epithelium, striking specific staining was localized to the nucleus and cytoplasm of the columnar epithelial cells lining the crypts (Fig. 3, D–I).

Tissue metabolite levels. Animals maintained on standard diets were weighed before sample collection (Table 1); apart from the expected sexual dimorphisms, there were no significant differences between the different genotypes.

In the Khk-A-expressing tissues heart and eye (Table 1) and brain, there was no significant difference in glucose or fructose levels among the three genotypes. Neither sugar was present at detectable levels in brain. In kidney (Khk-C expressing), glucose levels were again indistinguishable in wild-type and Khk<sup>3a/3a</sup> animals (Table 1). Fructose levels were, however, approximately fourfold higher in Khk<sup>3a/3a</sup> than in wild-type kidneys (<i>P < 0.05</i>). Sorbitol levels in brain, heart, and eye were below detectable limits in Khk<sup>3a/3a</sup>, heterozygous, and wild-type samples. Sorbitol was increased in the kidneys of Khk<sup>3a/3a</sup> compared with wild-type animals, but this did not reach statistical significance (Table 1).

Plasma biochemical analytes. The biological function of the widely expressed Khk-A isoform is undefined, although its kinetic parameters suggest that fructose is most likely not a physiological substrate. In a search for clues to such function, a range of standard biochemical analytes in plasma samples were measured (Table 2). None of these displayed significant differences between Khk<sup>3a/3a</sup> homozygous, heterozygous, and wild-type samples. Comparison of male and female samples revealed significant differences (<i>P < 0.05</i>) for total cholesterol, HDL cholesterol, and triglycerides.

In fasted Khk<sup>3a/3a</sup> animals, plasma creatinine was higher than control animals, but this did not reach statistical significance (Table 2). Plasma fructose levels were below detectable limits (not shown); these animals’ diets were not fructose supplemented.

Given the largely negative biochemical assay results, the feasibility of using plasma for a future metabolomic approach to identifying alterations of other low-molecular-weight metabolites was also investigated. NMR spectroscopy is one of the most frequently used platforms for metabolomic studies (32). Data on 26 mice (12 female, 14 male, various Khk genotypes) discriminated male from female animals (see Supplemental Material). Glucose was higher in males (not significant), lipids were higher in males (<i>P < 0.0001</i>), and 3-hydroxybutyrate was higher in females (not significant). Although these sex-dependent differences in metabolic profile dominated the model, small differences were identified that correlated with Khk genotype. Given the mixed genetic background of these animals, larger-scale experiments aimed at detecting genotype-dependent alterations in metabolomic profile will optimally be performed after breeding onto a congenic background.

DISCUSSION

The generation of two Khk-deficient animal models and their validation at the DNA, RNA, and protein levels have been described here. Both Khk<sup>3a/3a</sup> and Khk<sup>3a/3a</sup> animals were outwardly healthy and indistinguishable from their wild-type littermates. Furthermore, the fertility of both male and female homozygotes for either Khk allele, and the ability to carry normally sized litters to full term, were apparent. Since fructose is the dominant carbohydrate component of semen (23), the question of whether Khk deficiency would have an impact on the metabolic capacity of spermatozoa, and hence male fertility, had been a concern. However, spermatozoa are believed to metabolize fructose via hexokinase, and even though fructose predominates in semen glucose is preferentially utilized when available, because of its lower <i>Km</i> (23, 28).

Analysis of the function of the A isoform of Khk, weakly expressed in a wide range of tissues, has been difficult, largely because of its low level of expression and the lack of good immunologic reagents. Indeed, in Khk-A-expressing tissues, cellular and subcellular localization have not been amenable to immunohistochemical analysis, because of substantial nonspecific antibody cross-reactivity. Furthermore, although it was
Fig. 3. Immunohistochemistry on Khk-C-expressing tissues from Khk$^{Δ/Δ}$ and control animals. A–C: liver from homozygous Khk$^{Δ/Δ}$, heterozygous Khk$^{Δ/+}$, and wild-type Khk$^{+/+}$ animals. D–I: small intestine from the same animals, at low (D–F) and high (G–I) magnification.
shown by Western blotting that Khk<sup>3a/3a</sup> homozygous tissues lack the 30-kDa Khk protein, this was not confirmed by a corresponding loss of enzyme activity. Nonetheless, it is reasonable to infer that such an enzyme deficiency is present in Khk<sup>3a/3a</sup> animals, from the combined evidence of RNA transcript and Western blot analysis.

In tissues such as liver, kidney, and small intestine, which normally express high levels of Khk-C, interpretation is easier. By immunohistochemistry, these tissues appear indistinguishable between wild-type and heterozygous tissue extracts, it would appear that background contributed by other enzymes prevented the specific detection of the low level of Khk-C, even although a hexokinase inhibitor, N-acetylgalcosamine, was included (40).

Brain is another Khk-A-expressing tissue in which Khk activity has been previously assessed by others, again with conflicting results; activity has either been undetectable (1) or attributed to hexokinase (24) or ketohexokinase (4). Given the kinetic parameters of Khk-A, these studies did not employ suitable assay conditions. Without the means to effectively block other fructose-metabolizing enzymes, then, measurement of specific Khk-A activity in peripheral crude tissue extracts remains elusive.

Tissue fructose concentrations were higher in Khk<sup>ΔΔ</sup> than wild-type kidneys. Khk-C is highly expressed in the straight segment of the proximal renal tubule (13, 19). High-fructose diets have been implicated in the development of serious renal pathology. Some of these effects are probably systemically mediated: most dietary fructose is metabolized in the liver, causing rapid ATP depletion, which in turn increases AMP deaminase activity, raising circulating uric acid levels. This hyperuricemia is implicated in renal microvascular changes, with glomerular hypertension and hypertrophy, and may exacerbate preexisting renal pathology (11, 14, 27, 30). Recent work suggests that a high-fructose diet can induce hyperten-

### Table 1. Tissue metabolite levels

<table>
<thead>
<tr>
<th>Animal Weight, g</th>
<th>Glucose, mM</th>
<th>Sorbitol, mM</th>
<th>Fructose, mM</th>
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<tr>
<td><strong>Eye—Khk&lt;sup&gt;3a&lt;/sup&gt;</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>3a/3a (8 F)</td>
<td>25.4 (0.44)</td>
<td>2.65 (0.38)</td>
<td>0.123 (0.062)</td>
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<tr>
<td>+/+ (8 F)</td>
<td>24.9 (1.29)</td>
<td>2.41 (0.86)</td>
<td>0.107 (0.044)</td>
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<tr>
<td>3a/+ (8 F)</td>
<td>25.5 (3.06)</td>
<td>2.64 (0.38)</td>
<td>0.107 (0.070)</td>
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<tr>
<td>3a/3a (8 F)</td>
<td>1.13 (0.57)</td>
<td>Bg</td>
<td>Bg</td>
</tr>
<tr>
<td>+/+ (8 F)</td>
<td>1.91 (0.65)</td>
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<td>Bg</td>
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<tr>
<td>3a/+ (8 F)</td>
<td>1.42 (0.67)</td>
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<tr>
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<tr>
<td>+/+ (3 M)</td>
<td>31.5 (1.57)</td>
<td>8.05 (1.58)</td>
<td>1.41 (0.12)</td>
</tr>
<tr>
<td>D/Δ (3 M)</td>
<td>35.3 (0.40)</td>
<td>6.96 (1.50)</td>
<td>1.09 (0.24)</td>
</tr>
<tr>
<td>+/+ (3 F)</td>
<td>24.3 (1.29)</td>
<td>3.90 (0.33)</td>
<td>1.78 (0.24)</td>
</tr>
<tr>
<td>Δ/Δ (3 F)</td>
<td>20.5 (1.50)</td>
<td>4.32 (0.17)</td>
<td>3.27 (0.11)</td>
</tr>
</tbody>
</table>

**Pooling female and male samples (as only 3 in each class for Khk<sup>3a/3a</sup> animals)**

| +/+ (3 M, 3 F)        | 27.9 (4.15) | 5.96 (1.73)  | 2.18 (1.2)   |
| Δ/Δ (3 M, 3 F)        | 27.9 (1.86) | 5.64 (1.73)  | 2.65 (0.38)  |

Values are means (SD); metabolite values are expressed as μmol/g wet tissue. Khk, ketohexokinase; F, female; M, male; Bg, levels indistinguishable from assay background. *P < 0.05.

### Table 2. Plasma biochemistry in Khk<sup>3a/3a</sup> and Khk<sup>3</sup> animals

<table>
<thead>
<tr>
<th>Insulin, ng/ml</th>
<th>Creatinine, μM</th>
<th>Glucose, mM</th>
<th>Cholesterol, mM</th>
<th>HDL Cholesterol, mM</th>
<th>LDL Cholesterol, mM</th>
<th>Triglycerides, mM</th>
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<tbody>
<tr>
<td>Khk&lt;sup&gt;3&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3a/3a (5 F)</td>
<td>0.91 (0.18)</td>
<td>15.6 (7.5)</td>
<td>19.9 (1.9)</td>
<td>2.81 (0.47)</td>
<td>1.85 (0.41)</td>
<td>0.47 (0.08)</td>
<td>1.43 (0.44)</td>
</tr>
<tr>
<td>+/+ (4 F)</td>
<td>1.06 (0.47)</td>
<td>18.0 (4.0)</td>
<td>19.1 (3.5)</td>
<td>3.39 (0.97)</td>
<td>2.41 (0.75)</td>
<td>0.58 (0.16)</td>
<td>1.51 (0.46)</td>
</tr>
<tr>
<td>3a/+ (2 F)</td>
<td>1.0 (0.21)</td>
<td>16.9 (6.4)</td>
<td>16.3 (5.2)</td>
<td>2.72 (0.12)</td>
<td>2.27 (0.14)</td>
<td>0.64 (0.09)</td>
<td>1.55 (0.79)</td>
</tr>
<tr>
<td>3a/3a (8 M)</td>
<td>n/d</td>
<td>12.5 (5.3)</td>
<td>22.5 (7.5)</td>
<td>4.32 (0.96)</td>
<td>2.94 (0.49)</td>
<td>0.67 (0.17)</td>
<td>1.97 (0.49)</td>
</tr>
<tr>
<td>+/+ (8 M)</td>
<td>n/d</td>
<td>14.1 (5.4)</td>
<td>20.2 (5.1)</td>
<td>3.84 (0.44)</td>
<td>2.68 (0.37)</td>
<td>0.60 (0.09)</td>
<td>1.70 (0.36)</td>
</tr>
<tr>
<td>3a/+ (8 M)</td>
<td>n/d</td>
<td>15.2 (6.5)</td>
<td>22.0 (3.3)</td>
<td>4.36 (0.69)</td>
<td>2.93 (0.31)</td>
<td>0.69 (0.15)</td>
<td>2.09 (0.52)</td>
</tr>
</tbody>
</table>

Khk<sup>3</sup> (fasted)

| +/+ (2 M, 3 F) | 0.730 (0.24) | 7.7 (3.2)   | 17.4 (6.6)      | 3.7 (0.4)           | 2.7 (0.3)           | 0.5 (0.1)        | 1.1 (0.4)    |
| Δ/Δ (2 M, 3 F) | 0.887 (0.41) | 14.2 (5.5)  | 16.3 (8.7)      | 3.5 (1.0)           | 2.5 (0.7)           | 0.5 (0.2)        | 1.3 (0.7)    |

Khk<sup>3</sup> (nonfasted)

| +/+ (3 F)      | 17.1 (4.4)   | 19.1 (6.5)  | 3.1 (1.1)       | 1.9 (0.75)          | 0.6 (0.14)          | 1.5 (0.65)       | 209 (57)     |
| Δ/Δ (2 F, 1 M) | 17.1 (2.6)   | 18.8 (5.8)  | 3.6 (1.3)       | 2.3 (0.86)          | 0.5 (0.15)          | 1.3 (0.47)       | 294 (43)     |

n/d, Not determined.
sion, by altering salt absorption in the small intestine and kidney (33). Elevated postprandial peripheral blood fructose levels can also have a direct intrarenal impact due to metabolism in the kidney, causing an inflammatory response and renal vasoconstriction (10, 21). In contrast, the baseline studies described here were performed on animals maintained on standard chow, containing very little fructose or sucrose. The elevated fructose levels observed in the kidneys of Khk^{+/−} animals may reflect fructose generation within the kidney itself from glucose, through its metabolism to sorbitol and then fructose via the polyl pathway. This fructose could then accumulate to higher levels in Khk-deficient kidneys. However, this mechanism seems uncertain at present; although aldose reductase is reported to be present in the renal cortex, its level and activity of the polyl pathway are highest in the renal medulla, where sorbitol has an osmoregulatory role (9). Since Khk is located mostly in the renal cortex (13), the changes in fructose levels in Khk^{−/−} animals might relate to lower levels of polyl pathway activity in the cortex.

Although no other statistically significant changes in metabolite levels were observed in the knockout models, it is important to note that the animals under study had a mixed genetic background, which may limit the ability to detect subtle differences. Further studies in congenic animals are therefore needed; the modest elevation of renal fructose levels observed in Khk^{−/−} animals also requires verification in congenic animals. The statistically significant differences between males and females for some metabolites were as expected (18). In this study, the animals’ dietary carbohydrate source was primarily from wheat, barley, and maize. Further work is ongoing to examine the effects of high-fructose diets; in previous work from wheat, barley, and maize. Further work is ongoing to examine dietary fructose in relation to metabolic syndrome, this could be an important pointer toward the likely utility of Khk inhibitors to counter the corresponding human disease.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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