



## **Acute detachment of hexokinase II from mitochondria modestly increases oxygen consumption of the intact mouse heart**

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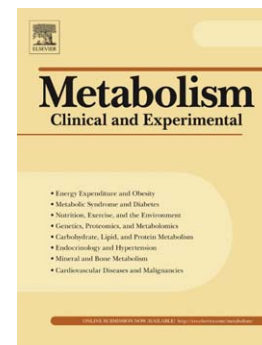
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## Acute detachment of hexokinase II from mitochondria modestly increases oxygen consumption of the intact mouse heart

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Running title: Mitochondrial hexokinase II and cardiac metabolism

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**Abstract**

*Objective.* Cardiac hexokinase II (HKII) can translocate between cytosol and mitochondria and change its cellular expression with pathologies such as ischemia-reperfusion, diabetes and heart failure. The cardiac metabolic consequences of these changes are unknown. Here we measured energy substrate utilization in cytosol and mitochondria using stable isotopes and oxygen consumption of the intact perfused heart for 1) an acute decrease in mitochondrial HKII (mtHKII), and 2) a chronic decrease in total cellular HKII.

*Methods/Results.* We first examined effects of 200 nM TAT (Trans-Activator of Transcription)-HKII peptide treatment, which was previously shown to acutely decrease mtHKII by ~30%. In Langendorff-perfused hearts TAT-HKII resulted in a modest, but significant, increased oxygen consumption, while cardiac performance was unchanged. At the metabolic level, there was a nonsignificant ( $p=0.076$ ) ~40% decrease in glucose contribution to pyruvate and lactate formation through glycolysis and to mitochondrial citrate synthase flux ( $6.6\pm1.1$  vs  $11.2\pm2.2\%$ ), and an 35% increase in tissue pyruvate ( $27\pm2$  vs  $20\pm2$  pmol/mg;  $p=0.033$ ). Secondly, we compared WT and HKII<sup>+/-</sup> hearts (50% chronic decrease in total HKII). RNA sequencing revealed no differential gene expression between WT and HKII<sup>+/-</sup> hearts indicating an absence of metabolic reprogramming at the transcriptional level. Langendorff-perfused hearts showed no significant differences in glycolysis ( $0.34\pm0.03$   $\mu\text{mol/min}$ ), glucose contribution to citrate synthase flux ( $35\pm2.3\%$ ), palmitate contribution to citrate synthase flux ( $20\pm1.1\%$ ), oxygen consumption or mechanical performance between WT and HKII<sup>+/-</sup> hearts.

*Conclusions.* These results indicate that acute albeit not chronic changes in mitochondrial HKII modestly affect cardiac oxygen consumption and energy substrate metabolism.

**Key words:** oxygen consumption; cardiac metabolism; hexokinase II; stable isotopes

## 1. Introduction

The glycolytic enzyme hexokinase (HK) facilitates the immediate phosphorylation of glucose upon entering the cell, thereby trapping glucose within the cell. The heart mainly contains the isoforms HK I and II, in approximately equal amounts [1]. HKI is present in almost all cells, mainly localized at the outer mitochondrial membrane, and hardly regulated by hormonal or metabolic signals. In contrast, HKII is mostly present in insulin-sensitive tissue (skeletal muscle, adipose tissue, heart) with its activity, expression and cellular localization (cytosolic or mitochondrial) highly regulated by hormonal or metabolic signals, and responsive to pathological conditions such as diabetes, heart failure, cancer and ischemia-reperfusion (IR) [2-7].

It remains unclear, however, what the metabolic consequences are of decreased mitochondrial HKII (mtHKII) or decreased total cardiac HKII on energy substrate selection and oxygen consumption within the intact heart. Fueger et al [8, 9] did show a reduced cardiac glucose uptake during exercise or an insulin clamp in *in vivo* HKII<sup>+/-</sup> as compared to wild-type hearts, but cellular glucose metabolism was not further studied. Cellular studies have suggested increased activity of HK when bound to mitochondria, due to privileged access to mitochondrially-produced ATP [10]. Binding of HKII to mitochondria resulted in increased glycolysis in isolated cell cultures provided with glucose-only substrate, whereas cytosolic HKII did not contribute to glycolysis [11, 12]. Whether these data would directly translate to similar effects (i.e. decreased glycolysis and/or glucose oxidation with decreased mtHKII) in the intact heart with high energy turnover and supplied with several substrates remains to be examined.

In addition, it is now well established that reductions in mtHKII makes the heart sensitive to IR injury [5, 13, 14]. Several mechanisms have been proposed to explain how diminished mtHKII in tissues or organs can increase sensitivity to IR damage [5, 12, 14]. One putative mechanism figuring prominently in IR injury, is the activity of the respiratory chain, activation of which often results in increased IR injury [15, 16]. No information is currently available whether decreased cardiac mtHKII,

either acutely or chronically, actually results in increased respiratory activity (oxygen consumption) within the intact heart. Accordingly, herein we examine the cardiac metabolic consequences of alterations in expression and cellular localization of HKII. Cardiac oxygen consumption and energy substrate metabolism in cytosol and mitochondria were determined in isolated heart models during perfusion conditions that were previously shown to have reduced mtHKII, i.e. 1) acutely through peptide treatment disrupting mtHKII binding in the presence of glucose, lactate and pyruvate [17], or 2) chronically through deletion of one HKII copy in the presence of glucose, lactate, pyruvate, insulin and fatty acids (FFA) [17, 18]. Cardiac metabolism of both heart models was determined using either stable isotope labeled glucose or stable isotope labeled palmitate to assess relevant metabolic fluxes using mass isotopomer distribution analysis [19]. To further examine whether cardiac metabolism is sensitive to the total amount of HKII chronically present, metabolic programming at the transcriptional level was compared between HKII<sup>+/-</sup> and WT hearts using RNA sequencing.

In the present study we hypothesize that within the intact heart 1) acute decreases in mtHKII are associated with decreased glucose breakdown and increased oxygen consumption, 2) chronic decreases in total and mtHKII result in altered cardiac metabolism (glucose and fatty acid breakdown), oxygen consumption, and differential (metabolic) gene transcription.

## 2. Methods

### 2.1. Animals

C57BL/6J HKII<sup>+/-</sup> [20] and their wild-type (WT) littermates were obtained from the in house breeding facility, as reported before [17]. The wild-type (WT) mice in the TAT-HKII series were all C57BL/6J and obtained from Charles River. Experiments were performed with male mice between 2-4 months old. All experiments were approved by the animal ethics committee of the Academic Medical Center, Amsterdam, The Netherlands. Although the experimenter at the day of experiment was aware of genotype and peptide treatment (this was inherent to the execution of the experiment because the experimenter had to select the animals from the breeding facility and make the peptide solution), all results assessed (metabolic, mechanical) were performed in a blinded fashion.

### 2.2. Heart Perfusion

Mice were heparinized (15 IU) and anesthetized with pentobarbital (80 mg kg<sup>-1</sup>). Following tracheotomy, the mice were mechanically ventilated and a thoracotomy performed. The hearts were cannulated in situ with perfusion started before excision of the heart. Hearts were Langendorff-perfused at a constant flow (initial perfusion pressure 80 mm Hg) at 37°C with non-recirculating Krebs-Henseleit solution containing (mmol l<sup>-1</sup>) NaCl 118, KCl 4.7, CaCl<sub>2</sub> 2.25 (2.5 in the 3% albumin series to ensure equal free calcium), MgSO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25, KH<sub>2</sub>PO<sub>4</sub> 1.2, EDTA 0.5. and gassed with 95% O<sub>2</sub>/5% CO<sub>2</sub>. The perfusate was in-line filtered by a 0.45-μm filter. End-diastolic pressure (EDP) was set at ~4-8 mmHg using a water-filled polyethylene balloon inserted into the left ventricular (LV) cavity via the mitral valve. The hearts were continuously submerged in 37°C perfusate. LV developed pressure was calculated as the systolic pressure (P<sub>sys</sub>) minus EDP. The rate-pressure product (RPP, index of mechanical performance) was the product of the developed LV pressure and the heart rate. Following stabilization of LV pressure, all hearts were perfused for 30 min, starting at baseline (t=0

min). For the TAT-HKII peptide series, we used the identical substrate composition of our previous study demonstrating the TAT-HKII induced ~ 30% decrease in mitochondrial HKII [17], i.e. 11 mM glucose, 1 mM lactate, 0.1 mM pyruvate and 0.5 mM glutamine. For the HKII<sup>+/-</sup> series with ~ 50% less cardiac HKII in the in vivo condition [18], the isolated hearts were also perfused with in vivo-like substrates: 5.5 mM glucose, 1 mM lactate, 0.1 mM pyruvate, 0.5 mM glutamine, 100 mU/L insulin, 0.05 mM L-carnitine and 0.3 mM palmitate bound to 3% albumin. Throughout perfusion, effluent samples were collected for oxygen partial pressures (at baseline and 25 min perfusion for the TAT-HKII peptide series, and at 25 min perfusion for the HKII<sup>+/-</sup> series) and lactate and pyruvate concentration (at 25 min perfusion for both series). For the TAT-HKII peptide series an estimate of glucose uptake was derived from glucose measurements in the influent and effluent samples using a bloodgas analyser (RapidPoint400, Siemens). Influent samples were collected at the end of each experiment. TAT and TATHKII peptide were dissolved in perfusate Krebs-Henseleit solution and administered at 1% of total perfusion flow through a side-arm connected to a custom-made mixing chamber. Peptide administration was started at baseline (t= 0 min) perfusion and continued to the end of the 30 min perfusion period. At the end hearts were immediately frozen in liquid nitrogen, weighted and stored at -80 °C until further analysis.

For the TATHKII peptide series we restricted ourselves to similar substrate composition for which we had been able to demonstrate previously, with the elaborate and demanding technique of immunogold labeling of HKII in hundreds of EM images (Smeele Km et al, CircRes 2011), that the used dosage of 200 nm TATHKII reduced mtHKII by about 30%. For the partial KO we were not restricted to a specific substrate composition, because we had shown previously that the difference in HKII occurs both in isolated hearts perfused with or without insulin, or in the in vivo condition with fatty acids present (Wu R, Smeele KM et al, Circ Res 2011). Therefore, we choose for the most physiological composition of the perfusate, i.e. next to glucose-lactate-pyruvate, also the addition of

insulin and fatty acids. This allowed evaluation of the role of HKII for cardiac metabolism within a high metabolic contextance.

### 2.3 *Mass isotopomer distribution analysis*

The isotope distribution of lactate, pyruvate and citric acid cycle intermediates was determined in heart tissue freeze clamped after Langendorff perfusion. Heart tissue (~100 mg wet weight) was homogenized in 1 mL 8% (w/v) sulfosalicylic acid and 100  $\mu$ L of internal standard (0.2 mM 2-phenylbutyric acid) using an Ultra-Turrax and sonication or the TissueLyser (Qiagen). The resulting suspension was centrifuged (10 minutes, 12000 g), after which the pH of the supernatant was adjusted to 7 by adding 30% NaOH dropwise. The sample was then split in two. In half of the extract the keto groups were directly oximated by addition of 50  $\mu$ L 5M hydroxylamine hydrochloride and incubation at 60°C for 30 minutes. The other half of the extract was first reduced by 50  $\mu$ L 200 mM NaBH<sub>4</sub>, and incubated at room temperature for 30 minutes. After inactivation and neutralization using hydrochloric acid and KOH/MOPS, respectively, the sample was further treated with citrate lyase and hydroxylamine, to convert citrate into oximated oxaloacetate (750  $\mu$ L of 500 mM triethylamine pH 7.4, 100 mM MgSO<sub>4</sub>, 50 mM EDTA, 10  $\mu$ L 5M hydroxylamine hydrochloride and 2.5U citrate lyase). The mixture was sonicated and incubated for 5 minutes at 37°C after which the sample was acidified by adding 100  $\mu$ L saturated sulfosalicylic acid and centrifuged (10 minutes, 1200 g). Next, both samples were reacidified by adding 100  $\mu$ L 37% HCl, and saturated with salt by adding ~0.2 g NaCl. The organic acids were extracted twice by adding 3 mL ethylacetate. Both ethylacetate extracts were pooled and dried. The residue was derivatized by addition of 75  $\mu$ L N-tert-butyltrimethylsilyl-N-methyltrifluoroacetamide, and incubation at 80°C for 60 minutes. Within 24 hours, the sample (1  $\mu$ L) was analyzed by gas chromatography-mass spectrometry (GC-MS). Peak intensities were integrated, corrected for natural abundance, and used for the calculation of

contribution of glucose and palmitate to acetyl-CoA formation for citrate synthesis [19].

Concentrations of citric acid, 2-ketoglutaric acid, malic acid, lactic acid, succinic acid, pyruvic acid and fumaric acid were determined using 0.2 mM 2-phenylbutyric acid as internal standard.

All hearts in the TAT series were perfused with [U-<sup>13</sup>C<sub>6</sub>] glucose (initial molar percent enrichment (MPE): 99%; Cambridge Isotope Laboratories, Andover, USA ); for the HKII<sup>+/-</sup> hearts unlabelled glucose or palmitate were replaced by either [U-<sup>13</sup>C<sub>6</sub>] glucose or [U-<sup>13</sup>C<sub>16</sub>] palmitate (MPE:98%; Cambridge Isotope Laboratories, Andover, USA), respectively, to probe both carbohydrates (CHO) and long-chain fatty acid metabolism (PAL). Using the stable isotopes analysis we determined: 1) anaerobic glycolysis from the efflux rate of [U-<sup>13</sup>C<sub>3</sub>]-labeled lactate and [U-<sup>13</sup>C<sub>3</sub>]-labeled pyruvate derived from the breakdown of exogenous [U-<sup>13</sup>C<sub>6</sub>] glucose; 2) fractional enrichment of tissue pyruvate by <sup>13</sup>C glucose, using the molar percent enrichment of pyruvate for all 3 C atoms of pyruvate (MPE pyruvate (M3))[21]; 3) fractional contribution of CHO (glucose and lactate-pyruvate) to acetyl-CoA formation for citrate synthesis (CS; from the <sup>13</sup>C enrichment of the acetyl and oxaloacetate moiety of citrate), this contribution is defined as pyruvate decarboxylation to citrate (PDC/CS); and 4) fractional contribution of PAL to acetyl-CoA formation for citrate synthesis (PAL/CS).

#### 2.4. RNA isolation and RNA sequencing analysis

RNA was isolated from 6 WT and 6 HKII<sup>+/-</sup> hearts using QIAzol lysis reagent followed by cleaned up using the RNeasy kit. RNA samples were submitted to the Genomics Core Facility at the Icahn Institute and Department of Genetics and Genomic Sciences. cDNA libraries were prepared using the Illumina TruSeq RNA Library Preparation kit (#RS-122-2001). Samples were run on Illumina HiSeq 2000 at a read length of 100nt single end, and a depth of about 35 million per sample. Count files were generated by aligning the fastq files to the mouse genome mm10 (UCSC) with STAR and then using featureCounts to count overlaps with exons. Final counts were grouped at the gene level [22].

Outlier detection identified 2 samples (1 WT and 1 HKII<sup>+/-</sup>), which were removed from subsequent analysis. Differential expression analysis was conducted with R the packages limma (with voom transformation) [23] and DESeq2 [24](Love et al, 2014). Low count genes were removed in the limma analysis, genes were kept if they had at least one count per million in at least 5 samples. A false discovery rate (Benjamini-Hochberg) of 0.05 was chosen as the cut off.

## 2.5. Statistics

Values are presented as mean  $\pm$  SEM. Differences between TAT and TATHKII groups for mechanical performance and oxygen consumption were analysed by a two-way ANOVA for repeated measurements followed by contrast comparison (SPSS version 23). Differences between groups were analyzed with paired or unpaired Student's t-test when applicable. Differences were considered statistically significant at  $P < 0.05$ .

### 3. Results

#### 3.1. *Functional effects of acute detachment of HKII using TATHKII peptide treatment in ex vivo Langendorff hearts*

The impact of 25 min TATHKII peptide treatment was compared with that of control, the TAT peptide (Figure 1). Peak systolic ventricular pressure (Fig. 1A) and RPP (Fig. 1C) showed a moderate decrease over time in the control group, probably as a result of deterioration of the preparation over time. No changes in heart rate over time were observed in the control group (Fig. 1B). TATHKII also significantly reduced RPP during 25 min peptide perfusion. However, TATHKII was without significant effect on these changes in mechanical performance when compared to vehicle. In contrast, TATHKII treatment resulted in a significant, albeit modest, increase in oxygen consumption as compared to vehicle (Figure 1D). No differences in flow or heart weight were present between groups (Table 1). These data demonstrate that acute detachment of HKII from mitochondria in the intact heart increased oxygen consumption with no direct effect on mechanical performance.

#### 3.2. *Metabolic effects of acute detachment of HKII using TATHKII peptide treatment in ex vivo Langendorff hearts*

Next we evaluated whether acute detachment of HKII from mitochondria affected glucose metabolism. Lactate and pyruvate efflux rate (reflecting glycolysis from exogenous labeled glucose) was low in all hearts, probably due to the lack of insulin in these conditions (Figure 2A). TATHKII treatment was without significant effect on the rate of anaerobic glycolysis, albeit the rate was reduced by 43% ( $p=0.28$ ). From the MPE of pyruvate it can be concluded that exogenous glucose contributes 38% to endogenous pyruvate formation, the remainder probably coming from exogenous lactate and pyruvate (table 1). TATHKII peptide treatment was without effect on the MPE of pyruvate, i.e. the pyruvate formation from glucose. The relative contribution of carbohydrates to

citrate synthesis is depicted in Figure 2B. Carbohydrates contributed approximately 28% to the citrate synthase flux, with the contribution of lactate being 18% and that of glucose 10%. TAT-HKII treatment resulted in a trend towards a decrease of glucose contribution to citrate synthase flux ( $p=0.076$ ).

To further explore possible mechanisms for the increased oxygen consumption and reduced glucose metabolism in the TAT-HKII treated hearts, we examined glucose uptake and  $^{13}\text{C}$  intermediates in the two groups of hearts (table 2). TAT-HKII treatment was associated with a non-significant trend ( $p=0.131$ ) in decreased glucose uptake, and a significant increase in tissue pyruvate concentration.

### 3.3. *Functional effects of chronic partial HKII knockout in ex vivo Langendorff hearts*

Mechanical performance and oxygen consumption for WT and HKII<sup>+/-</sup> hearts are given in Figure 3. Peak systolic pressure, heart rate and RPP are not different between the genotypes. Flow and heart weight were also not different between the genotypes (Table 1). Cardiac oxygen consumption is also not affected by deletion of one HKII copy.

In these insulin and FFA perfused hearts (in addition to glucose, lactate, pyruvate and glutamine) mechanical performance is similar to hearts perfused without insulin and FFA (compare to Fig. 1), when comparing baseline conditions between WT and TAT groups. However, adding 100 mU/L insulin and 0.3 mM FFA show a non-significant trend ( $p=0.084$ ) in lowering cardiac oxygen consumption (Fig.1D vs Fig. 3D;  $7.6 \pm 0.6$  vs  $6.3 \pm 0.4$   $\mu\text{mol}/\text{min}/\text{g}_{\text{ww}}$ , respectively).

### 3.4. *Metabolic effects of partial HKII knockout in ex vivo Langendorff hearts*

Anaerobic glycolysis rates in these hearts perfused with additional insulin and FFA was much higher ( $0.36 \pm 0.02$   $\mu\text{mol}/\text{min}$ ; Fig. 4A) than in the hearts perfused without insulin and FFA ( $0.07 \pm 0.03$   $\mu\text{mol}/\text{min}$ ; Fig. 2a), likely due to the presence of the relative high insulin concentration. Partial deletion of HKII was, however, without effect on anaerobic glycolysis rate. Glucose enrichment of

tissue pyruvate (table 1) was not significantly higher than in the hearts perfused without insulin and FFA, and was not affected by genotype ( $p=0.22$ ). The relative contribution of glucose and palmitate to acetyl-CoA formation for citrate synthesis in TCA cycle is shown in Fig. 4B and 4C, respectively. Despite the presence of FFA (known to inhibit glucose metabolism), carbohydrate contribution to citrate synthesis was again much higher ( $61.4 \pm 6.9\%$ ) in these insulin-perfused hearts as compared to hearts perfused without insulin ( $28.4 \pm 4.5\%$ ; Fig 2B). However, the contribution of glucose, and lactate and pyruvate, to citrate synthesis was not affected by the partial deletion of HKII. Correspondingly, palmitate contribution to acetyl-CoA formation for citrate synthesis was also similar between the two genotypes (Fig. 4C).

### 3.5. *Consequences of partial HKII knockout on the cardiac transcriptome*

In order to study the consequences of deletion of one HKII copy at the transcriptome level, we performed RNAseq analysis of WT and HKII<sup>+/-</sup>-hearts. Analysis of differentially expressed genes revealed a small impact of the partial HKII deletion, with only the decrease in HKII expression reaching the FDR cut off of 0.05 in both limma and DESeq2 (Table 3, showing the first 5 genes with largest tendencies of being differentially expressed between the genotypes). In DESeq2, two more genes, Antxr2 and Kbtbd12, were differentially expressed with small log2 fold changes (0.24 and 0.31, respectively). Given the lack of previous association of these genes with cardiac metabolism and the overall small changes in gene expression in the HKII<sup>+/-</sup>-hearts, the identification of these transcripts as differentially expressed is likely spurious.

#### 4. Discussion

Although it is well recognized that the degree of mitochondrion-HKII association is sensitive to many cardiac pathologies and interventions (IR, cardioprotection, insulin treatment, diabetes, hypertrophy, heart failure [5]), the metabolic consequences for the intact heart are, surprisingly, to a large extent unknown. We now report that 1) acute disruption of HKII from mitochondria in intact hearts is associated with an increase in oxygen consumption and a nonsignificant trend of decreased glucose contribution to citrate synthase flux; 2) chronically reduced HKII (including reduced mtHKII) is not associated with alterations in cardiac oxygen consumption, metabolism, or metabolic reprogramming at the transcriptional level of the intact heart not cause.

##### 4.1. *Acute decreases in mitochondrial hexokinase II and cardiac metabolism*

We have demonstrated that acute detachment of HKII from mitochondria increases cardiac oxygen consumption. We are unaware of other studies that have specifically examined the effects of mitochondria-HKII association on oxygen consumption. Our data indicates that HKII attachment to the mitochondria slows down mitochondrial respiration in a functional, working organ. This is commensurate with our previous observations that short periods of ischemia, able to induce cardioprotection and associated with increased mtHKII [25-27], result in slower mitochondrial activation of oxygen consumption [28, 29]. This slowing down was only observed with glucose, and not with lactate or pyruvate substrates, suggesting a glycolytic-origin, possibly HKII, of this effect. Since cardioprotection is associated with increased glucose metabolism [30], it is then suggested that increased glucose metabolism results in attenuated mitochondrial function. Indeed, in the present study increased binding of HKII to mitochondria was associated with a trend for increased glucose metabolism. It is generally believed that the physical binding of HK to mitochondria increases HK activity through decreasing its  $K_m$  for ATP, relieve of allosteric inhibition by G6P and glucose-1,6-

biphosphate, and preferential access to mitochondrial produced ATP [2, 31, 32]. HKII binding to mitochondria promoted glycolysis in CHO cells [11]. Increased glucose metabolism could potentially decrease mitochondrial oxygen consumption because of the higher P/O ratio for glucose relative to other substrates (less oxygen needed per ATP produced), or because of increased transport of cytosolic NADH into the mitochondria, which transport may impair oxygen consumption [33]. Alternatively, the increased oxygen consumption with similar cardiac function could also suggest a lower cardiac efficiency. Although care should be taken for over interpretation, the observed non-significant trend for decreased glucose uptake and increased tissue pyruvate concentration with less mtHKII, indicates that the decrease in glucose metabolism with detachment of mtHKII is controlled by impairment of glucose uptake and pyruvate oxidation within the intact heart. The increased oxygen consumption is then likely a result of a decrease in glucose metabolism, which, because no changes in mechanical work were observed, is then compensated with increased FA oxidation with its consequently higher oxygen need to provide similar ATP.

#### 4.2. *Chronic decreases in (mitochondrial) hexokinase II and cardiac metabolism*

In contrast to the acute manipulation of mitochondrial HKII, we observed no changes in oxygen consumption and metabolism in the partial HKII knockout heart, which we previously showed to have 50% less total and mitochondrial HKII [18]. Since chronic reductions in proteins are notorious for inducing compensatory alterations in other genes/proteins, possibly explaining the lack of changes in cardiac metabolism and oxygen consumption with chronic reduction in HKII, we characterized the cardiac transcriptome through RNA sequencing. The absence of differential gene expression demonstrated that compensation at the level of gene expression was absent, negating a role for gene expression adaptation in this model. However, at this stage, we cannot exclude that adaptation did occur posttranscriptionally (proteins and its posttranslational modifications). That cardiac glucose metabolism was unaffected in these partial HKII knockout hearts, is in support of

previous findings using the similar partial HKII knockout and reporting unaltered cardiac glucose uptake during sedentary conditions in these hearts [8, 34].

#### 4.3. *No insulin versus high-dose insulin and cardiac glucose metabolism*

In the present study we used perfusion conditions with and without insulin, as dictated by our previous experimental conditions where effects of the HKII manipulations were already determined [17, 18]. The absence of insulin, despite glucose being present at 11 mM, resulted in a rather low rate of anaerobic glycolysis ( $0.07 \pm 0.03$   $\mu\text{mol}/\text{min}$ ) and glucose contribution to acetyl-CoA formation for citrate synthesis ( $11.2 \pm 2.2\%$ ), even when compared to other heart studies using glucose-only perfusates. Total carbohydrates (glucose, lactate, pyruvate) contribution to acetyl-CoA formation for citrate synthesis amounted to near 30%, suggesting that a major part of citrate synthesis was derived from endogenous FA oxidation from stored triglycerides. It was shown before that in conditions of glucose-only perfusate myocardial triglyceride turnover contributes significantly to energy production in isolated hearts [35]. The low rates of glucose metabolism are likely due to the presence of 1 mM lactate; physiological concentrations of lactate decrease glucose metabolism to a large extent [36]. The data suggest that in perfusion conditions with glucose and lactate both being present, insulin is needed to facilitate glucose metabolism in the isolated heart. In the presence of insulin and FFA in the perfusate, carbohydrates and FFA acids contributed for 60% and 15%, respectively, to citrate synthesis in the intact hearts. These values are commensurate with values reported before in the isolated working rat heart [21], although much higher contributions of fatty acids have also commonly been observed. The relatively low fatty acid contribution could be due to the relatively high insulin concentration (100mU/L), the presence of 1 mM lactate (physiological concentration, and often neglected in isolated heart studies), knowing that lactate inhibits fatty acid oxidation [37], and the low, but physiologically-relevant for the fed condition [38], fatty acid concentration (0.3 mM).

#### 4.4. Methodological considerations

In the current study the effects of modest changes (30-50% decrease) in HKII on cardiac metabolism were examined. These modest HKII changes probably partly explain why only modest or even no changes in cardiac metabolism were observed. Importantly, these modest changes in HKII mimic the changes in HKII that occur during pathophysiological interventions such as IR and ischemic preconditioning [24 -26], type I and type II diabetes [4], insulin treatment [24, 39], or cardiac remodeling during hypertrophy and heart failure [6, 40]. Were we to use a complete knockout of HKII or transgenic overexpression of HKII by more than a factor of 5, larger changes in metabolism would probably have occurred. However, we believe the imposed manipulations of HKII and their metabolic consequences in the current study are more relevant to the real life pathophysiological conditions.

*In the present work effects of acutely reduced mtHKII on cardiac metabolism were examined during perfusion with a specific substrate composition of 11 mM glucose, 1 mM lactate and 0.1 mM pyruvate. It is known that this mixture results in only small contributions of glucose to ATP production [36], as supported by our low rates of glycolysis and glucose contribution to citrate formation, possibly explaining that we observed only non-significant trends for TAT-HKII effects on glucose metabolism. Therefore, it would be of interest to examine glucose contribution to citrate in the absence of lactate, or in the presence of insulin in the TATHKII series. Under these metabolic substrate conditions, glycolysis and glucose contribution to citrate would be more prominent making it perhaps easier to obtain significant effects of partly decreasing mt-HKII. Further research is needed to examine these questions. Finally, myocardial oxygen consumption was increased with acute detachment of HKII from mitochondria, but not with chronic reductions in mtHKII. New research should be directed to examine whether the acute increase in oxygen consumption is only a reversible phenomenon at maintained lower levels of mtHKII, possibly explaining that in longer-term oxygen consumption normalizes again to pre-treatment levels.*

In conclusion, our data suggest that an acute, but not chronic, decrease in HKII binding to mitochondria increases cardiac oxygen consumption that is associated with a trend for decreased glucose contribution towards citrate synthesis in the mitochondria.

#### **Authors Contributions**

RN, SMH and CJZ contributed to the design and conduct of the study, data collection and analysis, data interpretation and manuscript writing; BL contributed to design and conduct of the study, data interpretation and manuscript writing; SD, JH, CA contributed to data collection and analysis, data interpretation and manuscript writing; CDR, ML, RW, RHH and MWH contributed to data interpretation and manuscript writing. All authors approved the final version of the manuscript.

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#### **Disclosure statement:**

No relevant disclosures exist for this work

**Table 1.** Physiological parameters of isolated mouse hearts and  $^{13}\text{C}$  enrichments of tissue pyruvate isolated from Langendorff-perfused mouse hearts perfused with  $[\text{U-}^{13}\text{C}_6]\text{glucose}$ .

	TAT	TATHKII	WT	HKII <sup>+/-</sup>
Heart weight (mg)	165±5	178±5	198±8	210±8
Coronary flow (ml/min/g <sub>ww</sub> )	11.6±0.8	11.8±0.9	10.3±0.5	10.5±0.9
MPE pyruvate (M3)	38.2±2.9	34.8±1.9	44.4±5.1	37.0±3.0

Values are means ± SE. (n=8/9 for TAT/TATHKII hearts and n=16/17 for WT/HKII+/- hearts)

**Table 2.** Glucose uptake and  $^{13}\text{C}$  intermediates of metabolism derived from  $^{13}\text{C}$  enrichment

	TAT	TATHKII	P value
Glucose uptake ( $\mu\text{mol}/\text{min}/\text{G}_{\text{ww}}$ )	1.20 ± 0.59	0.27 ± 0.18	0.131
Pyruvic acid	20 ± 2	27 ± 2	0.033*
Lactic acid	8301 ± 1251	6208 ± 691	0.135
Citrate	778 ± 58	843 ± 53	0.424
2-ketoglutaric acid	5 ± 0	6 ± 0	0.087
Malic acid	232 ± 37	183 ± 20	0.235
Succinic acid	566 ± 35	479 ± 28	0.068
Fumaric acid	29 ± 6	27 ± 4	0.761

Values are means ± SE. (n=8/9 for TAT/TATHKII hearts). Metabolites given by pmol/mg<sub>ww</sub>

**Table 3.** RNA sequence and differential expression gene analysis results for two different analysis approaches (Limma and DESeq2). The first five genes with the largest differences in expression between genotypes are displayed.

Limma analysis			DESeq2 analysis		
gene	Adjusted P	Fold Change	gene	Adjusted P	Fold Change
HKII	0.010	0.57	HKII	$1.41 \times 10^{-13}$	0.68
Kbtbd12	0.657	1.77	Antxr2	0.021	1.18
Mir22hg	0.964	0.77	Kbtbd12	0.021	1.24
Lonrf1	0.964	1.22	Gm7120	0.123	0.84
Slc7a1	0.964	1.32	Wee1	0.408	1.19

Values are derived from five WT and five HKII<sup>+/−</sup> hearts.

## Legends

- Figure 1:** TATHKII peptide effects on mechanical performance and oxygen consumption of isolated hearts. Langendorff-perfused hearts were treated for 25 min, starting from baseline, with 200 nM TAT (control group) or 200 nM TATHKII peptide, and effects on peak systolic left ventricular pressure (Peak<sub>sys</sub>; 1A), heart rate (1B), rate-pressure product (1C) and oxygen consumption (1D). (n=8/9 per group). \**P* < 0.05 vs baseline or TAT.
- Figure 2:** TATHKII peptide effects on cardiac metabolism in isolated hearts perfused with <sup>13</sup>C glucose, lactate and pyruvate. TATHKII effects on anaerobic glycolysis (2A) and the contribution of carbohydrates to citrate synthesis, as assessed by the PDC/CS ratio for carbohydrates, lactate and glucose (2C), are given. (n=8/9 per group).
- Figure 3:** Mechanical performance and oxygen consumption of HKII<sup>+/-</sup> and WT isolated hearts. Peak systolic left ventricular pressure (Peak<sub>sys</sub>; 1A), heart rate (1B), rate-pressure product (1C) and oxygen consumption (1D) for HKII<sup>+/-</sup> and WT hearts. (n=16/17 per group).
- Figure 4:** Cardiac metabolism in isolated HKII<sup>+/-</sup> and WT hearts perfused with <sup>13</sup>C glucose or <sup>13</sup>C palmitate, and lactate, pyruvate and insulin. Effects of HKII<sup>+/-</sup> on anaerobic glycolysis (4A), on the contribution of carbohydrates to citrate synthesis as assessed by the PDC/CS ratio for carbohydrates, lactate and glucose (4B), and on palmitate contribution to citrate synthesis (4C) are given. (n=7/8 per group).

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Figure 1

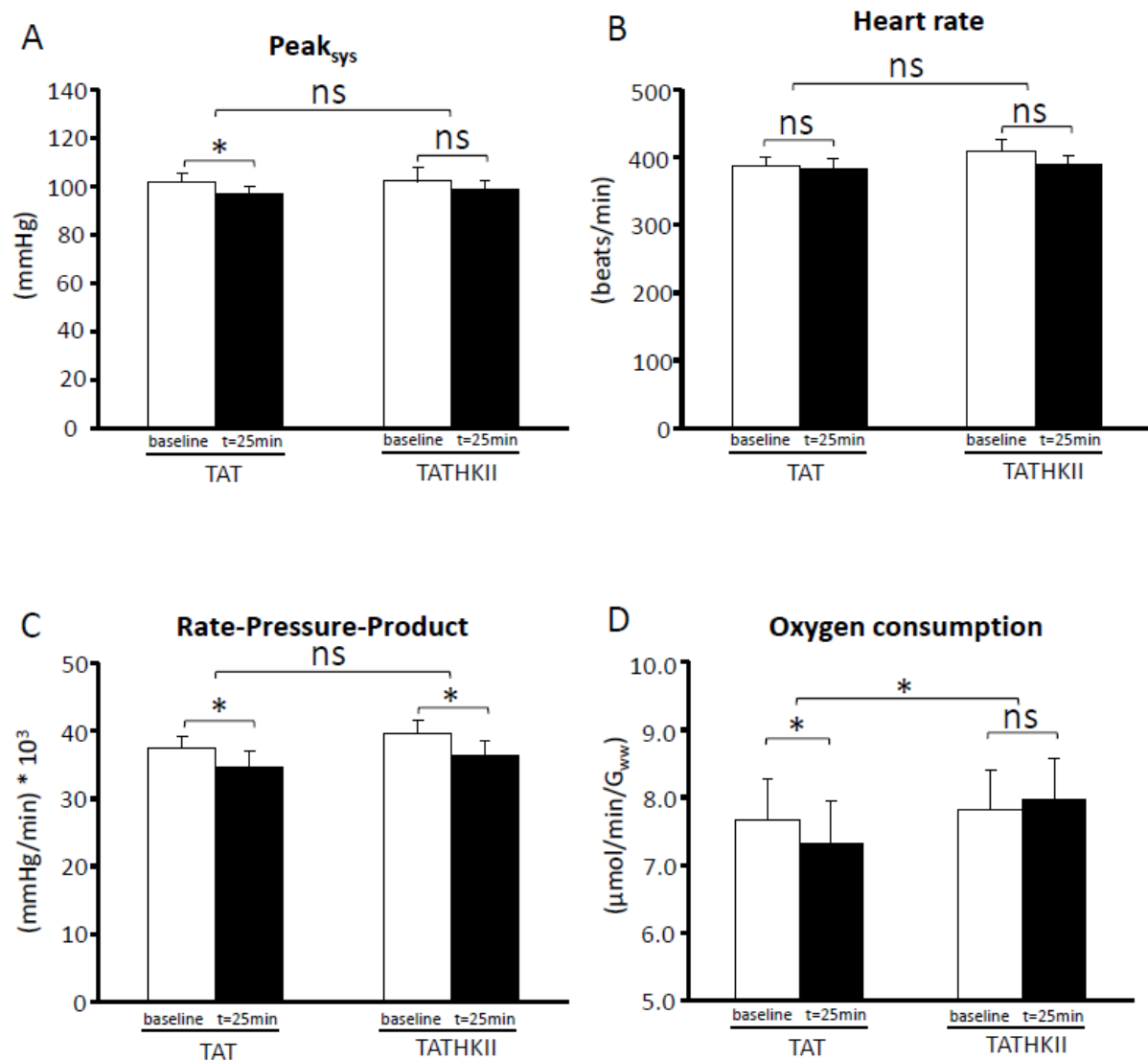


Figure 2

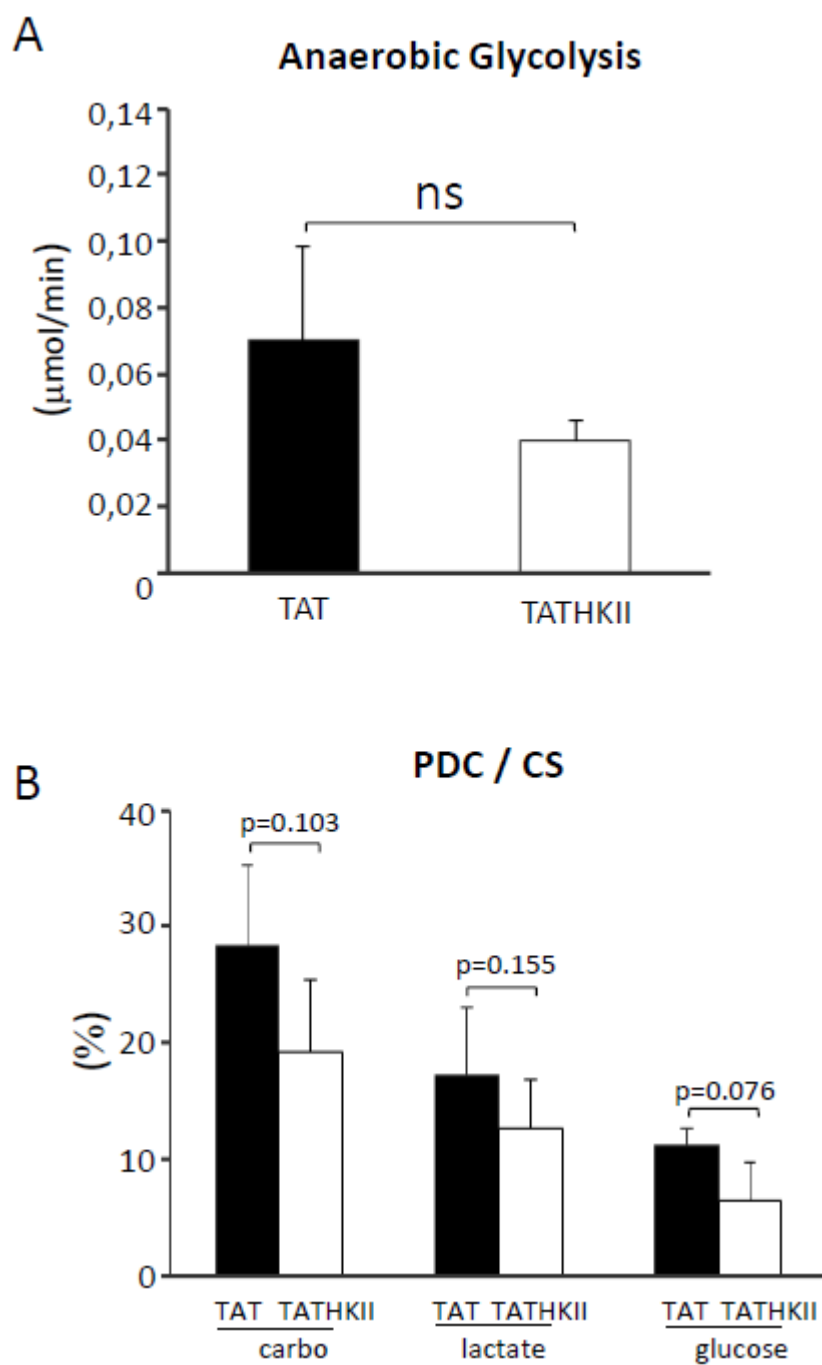


Figure 3

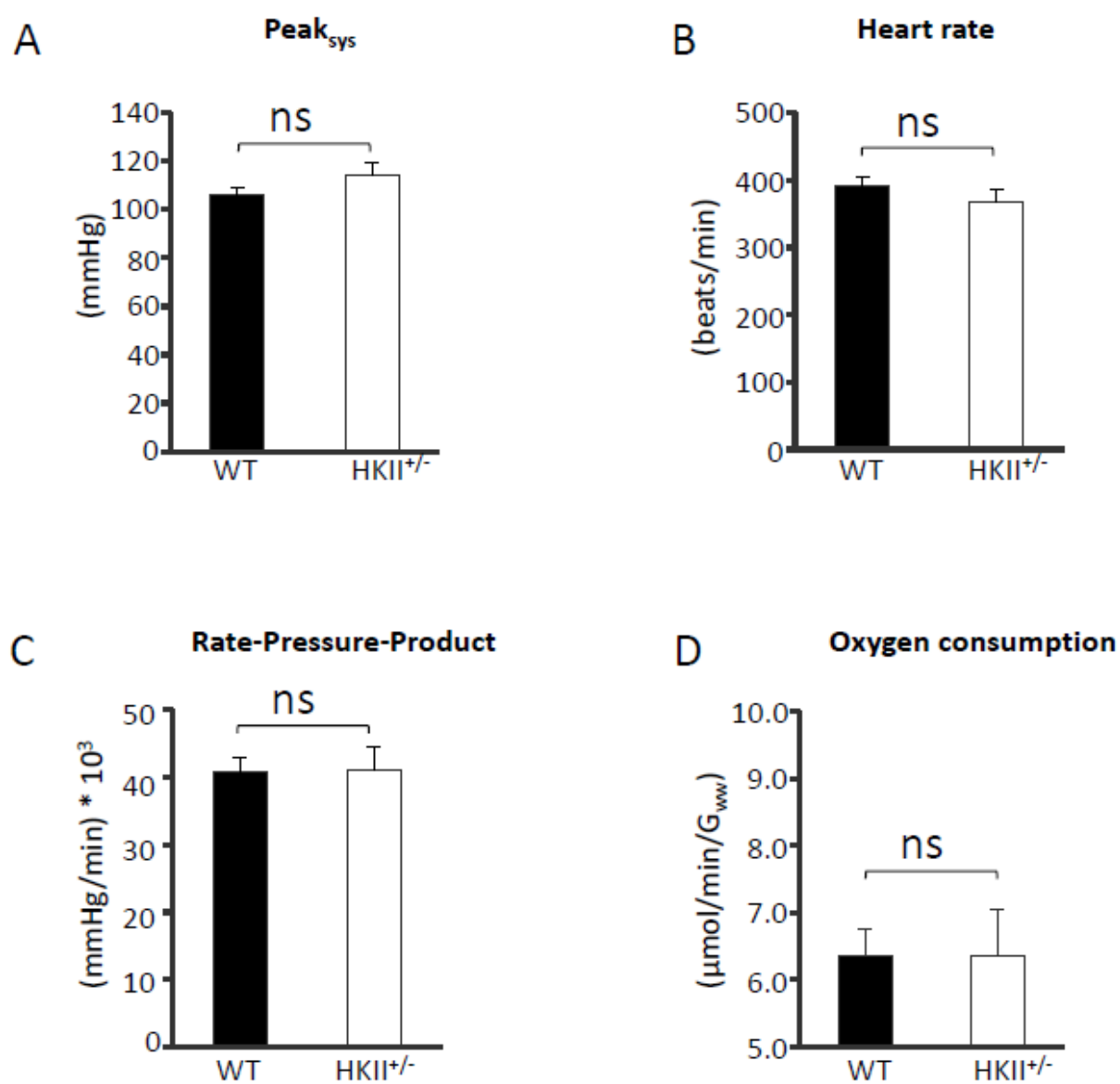
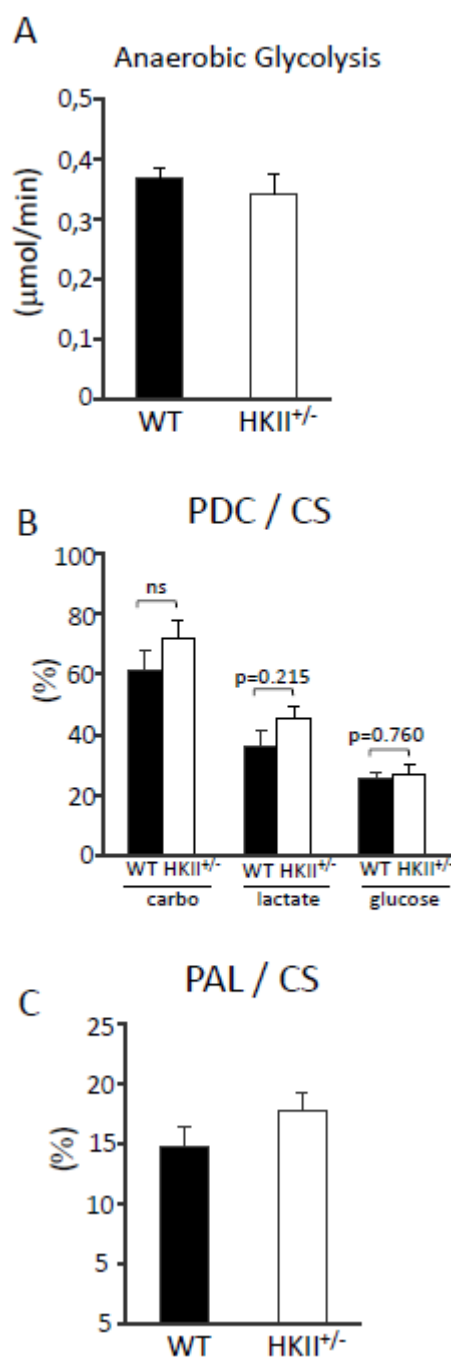


Figure 4



### Highlights

- Acute detachment of Hexokinase II from mitochondria increases cardiac oxygen consumption
- Acute detachment of hexokinase II from mitochondria trends to decrease glucose metabolism
- Chronic decrease in hexokinase II is without effect on cardiac metabolism
- 50% reduction in HK2 is not associated with metabolic transcriptional reprogramming