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Published in:
Journal of Applied Physiology

DOI:
10.1152/japplphysiol.00207.2018

Publication date:
2019

Document version
Peer reviewed version

A rapid radiochemical filter paper assay for determination of Hexokinase activity and affinity for glucose-6-phosphate (G6P)

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Abstract

Glucose phosphorylation by hexokinase (HK) is a rate-limiting step in glucose metabolism. Regulation of HK includes feedback inhibition by its product glucose-6-phosphate (G6P) and mitochondria binding. HK affinity for G6P is difficult to measure because its natural product (G6P) inhibits enzyme activity. HK phosphorylates several hexoses and we have taken advantage of the fact that 2-deoxyglucose-6-phosphate (2-DG-6P) does not inhibit HK activity. By this we have developed a new method for rapid radiochemical analysis of HK activity with 2-deoxyglucose (2-DG) as substrate, which allows control of the concentrations of G6P to investigate HK affinity for inhibition by G6P. We verified that 2-DG serves as a substrate for the HK reaction with linear time and concentration dependency as well as expected V_{max} and K_M. This is the first simple assay evaluating feedback inhibition of HK by its product G6P and provides a unique technique for future research evaluating the regulation of glucose phosphorylation under various physiological conditions.

New & Noteworthy

Traditionally, hexokinase activity has been analyzed spectrophotometrically where the product formation of G6P is analyzed by an indirect reaction coupled to NADPH formation during conversion of G6P to 6-P gluconolactone. By nature this assay prevents measurements of HK affinity for inhibition by G6P. We have developed a rapid radiochemical filter paper assay to study HK affinity for G6P by use of radiolabeled 2-DG as substrate, to study physiological regulation of HK affinity for G6P-induced inhibition.

Introduction. Glucose uptake in various tissues is regulated by glucose delivery, glucose transport across the membrane and intracellular phosphorylation (32). Although glucose phosphorylation can be limiting for glucose uptake under various physiological conditions (2), the regulation of glucose phosphorylation has received surprisingly little attention. Hexokinase (HK) catalyzes the irreversible reaction converting glucose to glucose-6-phosphate (G6P). Glucose phosphorylation is a potential rate-limiting step for glucose metabolism and Mammalians express four different isoforms of hexokinase (hexokinase I-IV) (37) while skeletal muscle expresses HK I and II (6, 11). HK is inhibited allosterically by G6P with a K_i for G6P in the physiological range (37). It has been known for many years that HK binds to mitochondria and it was more recently reported that HK II is phosphorylated by PKB at Thr^{473} (26). The translocation of HK to the mitochondria reduces the ability of G6P to inhibit enzyme activity. However, the fact that the product inhibits HK activity makes is difficult to study the physiological regulation of HK by G6P.

HK activity can easily be measured spectrophotometrically or fluorometrically (28). In this method G6P-dehydrogenase (G6P-DH) in the assay buffer converts the formed G6P to 6-Phosphogluconolactone in a reaction coupled to conversion of $NADP^+$ to NADPH; production of NADPH is measured to describe HK activity (28). Inclusion of G6P-DH in these methods is required for the formation of NADPH, the presence of G6P-DH also prevents the accumulation of G6P which otherwise would exert a negative feedback on HK enzyme activity. Radiolabeled glucose or ATP can also be used to measure HK activity where glucose and G6P can easily be separated. Sanderson et al. used ^{14}C-labelled glucose to measure HK activity and excluded G6P-DH from the assay buffer. In this study the authors demonstrated less inhibition of HK activity as G6P accumulated after insulin stimulation in skeletal muscles from lean but not from obese Zucker rats (28). This suggests that insulin

decreases HK affinity for G6P-induced inhibition, but the method does not allow to calculate K_i (inhibitory constant that is reflective of the binding affinity) for G6P-induced inhibition of HK. These data suggest that HK affinity for G6P is regulated in skeletal muscles and highlights that methodologies to investigate HK affinity for G6P are required.

Hexokinase phosphorylates a number of hexoses like 2-deoxyglucose (2-DG), but only G6P and a few other hexose 6-phosphates inhibit HK activity (36). Importantly, 2-deoxyglucose-6-phosphate (2-DG-6P) does not inhibit HK activity (37). Thus, 2-DG serves as ideal substrate for HK activity assay where the sensitivity for inhibition by G6P can be studied. Here we present a novel radiochemical filter paper assay for rapid determination of HK activity and affinity for inhibition by G6P. This method may not only be applicable for muscle physiology but also for cancer research as the malignant phenotype of cancer cells is associated with increased hexokinase expression and glycolytic flux (20).

Materials and methods

Experiments were conducted in Norway and Denmark. Experiments in Norway were approved by Norwegian authorities and experimental procedures in Denmark by Danish and Animal Experimental Inspectorate. All experiments were conducted in compliance with the European Union convention for protection of vertebra animals used for scientific purposes (Council of Europe 123, Strasbourg, France, 1985).

Animals. In Norway male Wistar rats were purchased from B & K Universal (Nittedal, Norway). In Denmark male Wistar rats and female C57B6 mice were purchased from Taconic (Ejby, Denmark).

Animals were kept in a room with 12:12-h light-dark cycle (light on/off, 6:00 AM/6:00 PM) and had free access to water and chow.

Tissue preparation and muscle incubation. Animals were anesthetized by intraperitoneal injection of pentobarbital sodium (10 mg/100 g body wt) and tissues were dissected. Tissue samples were homogenized directly (fresh tissue). In some studies, epitrochlearis and soleus were contracted or treated with insulin *in vitro* prior to analysis of HK activity. Information about experimental conditions is given in figure and table legends. For *in vitro* studies, muscles were dissected and suspended at approximate resting length in an incubation system containing Krebs-Henseleit buffer (KHB) as previously described (1). After 30 min preincubation, muscles were either rested for 30 min, stimulated by supraphysiological insulin concentration (10 mU/ml) or forced to contract by electrical stimulation. Muscle contraction was induced by electrical stimulation for 30 min consisting of 200-ms trains (100 Hz, 0.2 ms pulse duration, 10 V) delivered every 2 s, a protocol that previously has been shown to result in a robust increase in glucose uptake (1). During the entire incubation period the buffer was maintained at 30ºC and oxygenated with 95% O_2 and 5% CO_2. After incubation, muscles were harvested, blotted on filter paper and immediately homogenized.

Homogenization. Tissue was homogenized in ice cold buffer containing 250 mM sucrose, 20 mM Sodium-pyrophosphate, 5 mM HEPES (pH 7.4), 5 mM magnesium chloride, 1 mM DTT, 5% Dextran 70, 20 mM β-glycerophosphate, 10 mM sodium fluoride, 2 mM PMSF, 1 mM EGTA (pH 8.0), 10 µg/ml Aprotinin, 10 µg/ml Leupeptin, 2 mM sodium orthovanadate and 3 mM benzamidine for 2 x 30 s at 30 Hz using a TissueLyzer II (Qiagen, Hilden, Germany).

Homogenates were analyzed for total protein concentration by use of the bicinchoninic acid method (Thermo Scientific, Waltham, MA, USA).

A detailed list providing product no. and stock concentrations for reagents used in the homogenization buffer is given in Supplemental I (https://doi.org/10.5281/zenodo.3249956).

Filter paper HK activity assay. Experiments were initially performed in Eppendorf tubes and the reaction buffer with homogenate was spotted on DE81 anion exchanger filter paper (Whatman, UK, #3685 915) and washed as described below. To optimize and make the assay more efficient, the assay was changed to 96-wells PCR plates (Thermo Fisher Scientific, AB0600). Homogenates were diluted to ~ 1-2 mg protein/ml in homogenization buffer. 15 µl sample was added to a 96-well PCR plate placed on an ice cold aluminum plate. As controls 15 µl homogenization buffer, cold assay buffer (buffer blank), a standard pool and a blank sample (sample blank) was added to every assay plate. Kinase activity was started by adding 15 µl assay buffer in 1 sec intervals with a mutichannel pipette while the aluminum plate was placed on a 30°C heating block. The assay buffer contained 50 mM TEA (pH 7.4), 5 mM ATP, 5 mM magnesium chloride, 10 mM creatine phosphate, 2.4 µCi/ml ^{14}C-2-deoxyglucose or ^{3}H-2-deoxyglucose (Perkin Elmer) , 2 mM 2-DG, 0.05% BSA, 7 U/ml creatine kinase. In case HK activity was evaluated in the presence of G6P or 2-DG-6P, various concentrations were added to the assay buffer (see results for details). Kinase reaction was stopped at 30 °C after 15-25 min (depending on tissue and protein amount in the reaction) by adding 30 µl (1 vol) 90 % EtOH. The PCR plate was kept under gentle agitation (manual agitation) at room temperature for 30 sec to ensure full mixture and hence complete stop of kinase reaction. 20 µl were spotted in duplicates by a multichannel pipette on DE81 filter paper. The filter paper was subsequently washed 3 x 20 min in 300 ml 60% EtOH in a plastic box (31cm x 22 cm x 6 cm) to remove non-phosphorylated 2-DG and dried in a fume hood. In order to determine specific activity, assay buffer (2 x 1 µl and 2 x 2 µl) was spotted on the

dried filter paper and added in vials containing 4 ml scintillation fluid. Finally the dried DE81 paper was wrapped in plastic wrap and placed on a STORM cassette for 48 hours and analyzed on a STORM Phosphoimager. Hexokinase activity was calculated as amount of ^{14}C -2-DG6P or ^{3}H-2-DG6P captured in the DE81 filter paper and given relative to specific activity of the assay buffer. A step-by-step description of the assay is given in Supplemental II (https://doi.org/10.5281/zenodo.3254455).

Comparison of fluorometric and radiochemical assay for determination of HK activity.

Tissue was homogenized as described above and analyzed for HK activity by the new radiochemical filter paper assay and compared to the well-established fluorometric method.

For fluorometric determination of HK activity, 20 µl sample was loaded as triplicate on a microtiter plate and 200 µl reaction mixture was added. The reaction mixture contained 200 mM Tris buffer (Tris HCl, Calbiochem 648313 and Tris Base, Calbiochem 648311) (pH 8.0), 10 mM magnesium chloride (Sigma M2670), 12.5 mM Dithiothreitol (DTT) (Sigma D0362), 25 mM Glucose (Sigma G8270), 1 mM NADP, 11 U/ml G6PDH. The reaction was started by adding 10 µl ATP (100mM) (Sigma A 3377) and absorbance was measured at 340 nm as described by Lowry & Passoneau (18).

Effect of exercise training on HK activity: Quadriceps muscle samples from female WT mice (SV129/C57B6 mixed background) from a previous study (9) were investigated for HK activity by use of the new radiochemical filter paper assay and compared to the fluorometric method. Briefly, mice were housed in single cages for 4 weeks without (sedentary) or with (training) access to a running wheel. Mice were euthanized by cervical dislocation and muscle tissue was frozen in liquid nitrogen and stored at -80 °C. We have previously investigated these samples by western blotting and reported a ~3 fold increase in training-induced HKII protein expression (9).

Results.

In the new radiochemical filter paper assay glucose phosphorylated by HK to G6P will be isolated by binding to the filter paper (Figure 1). Initially we compared the new radiochemical filter paper assay to the established fluorometric method for determination of HK activity (Table 1). Immediately after euthanization, rat brain, heart, soleus muscle and extensor digitorum longus (EDL) muscle were homogenized. One portion of the homogenate was analyzed by the fluorometric assay while another portion from the same homogenate sample was analyzed by the new radiochemical filter paper assay. This enabled for direct comparison between these methods and revealed that the new filter paper assay yields comparable results to the established fluorometric assay (Table 1).

Based on multiple analyses intra-assay variation and inter-assay variation were calculated to be 5-10% and 10-16%, respectively. These were found to be comparable to other enzymatic assay such as the GS-activity assay. Collectively these data demonstrate that the filter paper assay shows sensitivity for detection of radiolabeled G6P that is comparable to the established fluorometric assay.

The fact that HK activity is inhibited by its product G6P precludes the use of glucose as substrate for determination of HK affinity for G6P. However, hexokinase phosphorylates a number of hexoses that do not inhibit HK activity and hence may serve as candidates to study alterations in affinity of HK activity for product inhibition by G6P (37). Therefore, we investigated 2-DG as a substrate for the filter paper assay and compared it to glucose (Figure 2A). HK activity in rat heart was assayed in the presence of increasing concentrations (0, 0.025, 0.05, 0.1, 0.2, 0.5, 1, 2 and 5 mM) of 2-DG and glucose, respectively (Figure 2A). Kinetic parameters were calculated by Lineweaver Burk Plots (Figure 2B-C) and are given in Table 2. Hexokinase enzyme reaction velocity was substantially

169 higher using 2-DG compared to glucose with a maximal velocity (V_{max}) of 14 and 6 μmol/g
170 protein/min, respectively. K_M values derived from Lineweaver-Burk plot revealed a higher value for 2-
171 DG compared to glucose (0.67 vs. 0.16 mM). These findings do not preclude kinetic studies of HK
172 activity with 2-DG as a substrate; instead the markedly higher HK activity in light of a higher K_M
173 demonstrate that 2-DG serves as optimal substrate to study kinetic properties of HK. Furthermore,
174 product formation (2-DG-6P) in rat heart increased linearly in a time- and concentration-dependent
175 manner (Figure 3A-B), validating the experimental settings of the radiochemical filter paper assay with
176 2-DG as a substrate.

177 Regular exercise training leads to a substantial increase in HK II expression and activity (35). In
178 order to verify that the new radiochemical filter paper assay is capable to detect alterations in HK
179 expression, we analyzed muscle sample from a previous mouse exercise training study (9).
180 Determination of HK by the fluorometric and filter paper assay revealed a comparable increase in HK
181 activity by exercise tranining (~2.7 fold increase for fluorometric assay and ~2.5 fold increase for the
182 filter paper assay) (Figure 3C). These data are in accordance with the markedly higher levels of HK
183 expression we previously have reported (9) and demonstrate that the filter paper assay shows sensitivity
184 for detecting differences in HK activity.

185 Given that majority of previous studies have investigated HK activation in frozen tissue, we
186 tested whether the radiochemical filter paper assay also can be used to investigate HK activity in frozen
187 tissue (Figure 3D). Rat soleus muscles were split in to two portions; one being homogenized as frozen
188 tissue and the other part being homogenized as fresh tissue. Homogenate from frozen and fresh tissue
189 was analyzed for HK activity by the fluorometric assay and the new filter paper assay. These data
190 demonstrate that the radiochemical filter paper assay can be used to investigate HK activity in frozen

191 tissue, albeit the HK activity generally tended to be a few percent lower in homogenate from frozen
192 tissue. Previous studies have reported HK binding to mitochondria as a potential mechanism for
193 reducing affinity for G6P-mediated inhibition (20, 26). In an attempt to maintain this interaction we
194 decided to perform subsequent analyses on fresh tissue.

195 Next, we studied HK activity in in the presence of increasing concentrations (0, 0.1, 0.2, 0.5 and
196 1 mM) of 2-DG-6P and G6P (Figure 4). This experiment reveals that increasing levels of G6P inhibit
197 HK activity with a 50 % reduction (K_i) at ~0.2 mM G6P, while a similar increase in 2-DG6P in the
198 assay buffer has no effect on HK activity. These findings verify that radiolabeled 2-DG can be used as
199 substrate without that the formed 2-DG-6P exerts a negative feedback on HK activity.
200 When assayed in the presence of increasing G6P concentrations, the physiological regulation of HK
201 affinity for G6P may be detected. Insulin and muscle contraction serve as main physiological stimuli to
202 increase muscle glucose uptake and a subsequent rise in intracellular G6P levels (2, 14). Despite
203 elevated G6P levels skeletal glucose flux increases, suggesting that the negative feedback G6P exerts
204 on HK activity is antagonized under these physiological conditions. In order to investigate whether
205 sensitivity of HK for G6P underlies physiological regulation rat epitrochlearis muscles were stimulated
206 *ex vivo* by insulin (10 mU/ml) or forced to contract for 30 min by electrical stimulation (Figure 5).
207 Maximal hexokinase activity (~1.8 μmol/g protein/min) was observed when assayed in the absence of
208 G6P with no effect of insulin stimulation or muscle contraction. HK activity decreased with increasing
209 G6P levels in a similar pattern in all three conditions and with a half-maximal inhibition (K_i) around
210 0.2 mM G6P. Thus, under the present experimental conditions HK affinity for G6P remained
211 unaffected by insulin stimulation and muscle contraction.

212

Discussion

Hexokinase is the classical example of an enzyme that is inhibited by its product (G6P) in a feedback dependent manner. However, regulation of HK activity is complex and HK translocates to mitochondria and becomes phosphorylated (26). Translocation of HK to mitochondria has obtained large interest, but the effect of this translocation on HK activity is poorly understood. There are indications that translocation of HK to the mitochondria reduces affinity for G6P-induced inhibition, but such analyses are hampered by the fact that HK is inhibited by its natural product G6P (37). Other proteins are regulated by G6P; e.g. the major regulatory mechanism for glycogen synthase (GS) is by changes in affinity for activation by G6P. This occurs via phosphorylation/dephosphorylation mechanisms (15, 17, 25). GS activation is normally reported as fractional activity, where activity at a physiological concentration of G6P is related to total activity measured with a high concentration of G6P sufficient to activate GS completely (12). Our idea was to establish a comparable assay where HK activities are measured without G6P (total activity) and with a physiological concentration of G6P to investigate changes in HK affinity for G6P-induced inhibition. Therefore, we have developed a simple and rapid method for measurement of G6P-induced inhibition of HK by using 2-deoxyglucose (2-DG) as substrate to avoid inhibition by the product. This method can be used to evaluate regulation of HK activity in various cell types and under various experimental conditions.

Here we present a rapid radiochemical filter paper assay that reveals HK activity measurements with a linear dose and time dependency comparable to the well-established fluorometric assay. Moreover, the use of radiolabeled 2-DG as a substrate in the filter paper assay allows to study the

sensitivity of HK for inhibition by its product G6P. We could demonstrate that increasing levels of G6P but not 2-DG6P inhibit HK activity, providing a model to study the regulation of HK sensitivity for G6P under various physiological settings.

Intracellular G6P serves as central regulator for skeletal muscle glycogen synthesis and should be considered together with the degree of dephosphorylation of glycogen synthase when evaluating the rate of glycogen synthesis (30). On the other hand G6P exerts a negative feedback on HK and the intracellular concentration of G6P must therefore be balanced between activation of glycogen synthase and inhibition of HK (19). We observed a half maximal inhibition (K_i) of HK activity at ~0.2 mM G6P, suggesting that HK was potently inhibited by levels of G6P thought to exist in skeletal muscle *in vivo*. Thus, previous studies have reported G6P concentrations within the range of 0.2 – 0.3 mM under resting conditions and increase above 1 mM in response to stimulation by insulin and muscle contraction (14, 19, 23, 24). The G6P concentrations applied in the current HK assay (0.1-1 mM) therefore reflect what could be expected in the physiological range. Increased glucose uptake and a subsequent rise in intracellular G6P associated with insulin stimulation and muscle contraction should consequently increase inhibition of hexokinase further, yet an increased flux is observed *in vivo* (2, 4, 27). This suggests the presence of mechanisms that uncouple the negative feedback associated G6P levels and HK activity *in vivo*. In contrast to our hypothesis, HK sensitivity for G6P in response to insulin stimulation and muscle contractions remained unaffected under the experimental conditions in the present study. Sanderson et al (28) have previously reported that insulin reduced G6P-induced inhibition in skeletal muscles, and other studies have proposed association of HK with mitochondrial membrane as a mechanism to regulate HK activity (3, 21, 37). Thus, once HK I and II are bound to outer mitochondrial membrane inhibition of enzyme activity by G6P is antagonized. It has been

reported that insulin stimulation (3, 37) and muscle contraction (13, 33) increase binding of HK II to the mitochondrial fraction. We used a homogenization buffer (containing 5% dextran 70 and 5 mM $MgCl_2$) and a procedure (brief homogenization of fresh tissue) that previously has been shown to maintain the integrity of the mitochondria (7, 8, 28, 34). Nevertheless we were unable to observe a regulation of HK inhibition in response to insulin stimulation or muscle contraction. Insulin and exercise have been shown to increase expression of HKII in human skeletal muscle whereas HKI remains largely unaffected in response to these stimuli (16, 31). Notably this isoform specific regulation was associated with increased HKII activity in soluble but not in the particulate fraction, highlighting the importance subcellular regulation when investigating the regulation of HK activity. In the present study HK enzyme activity was not investigated isoform specific in cellular sub-fractions, so we cannot rule out the possibility that regulation of HKII activity may have been overseen due to the crude measurement in the present study.

Hexokinase binds to the mitochondria (VDAC) via it N-terminal end and mutating the fifth amino acid histidine to proline completely abolish HK-2 binding to the mitochondria (5). The interaction between HK2 and VDAC is not characterized, but it tempting to speculate that the binding is rather loose and may be disrupted during homogenization. However, mitochondria can be isolated with attached HK (22) and it could therefore be speculated that isolation of mitochondrial fraction is required to study these alterations in G6P-mediated inhibition of HK activity. Indeed, Van Houten and colleagues reported an increase in HK activity in the mitochondrial fraction but not total fraction when rat gastrocnemius muscles were analyzed following swimming exercise until exhaustion (13). We suggest that other homogenization procedures or studies in saponised muscle bundles should be investigated.

The interaction between HKII and mitochondria (VDAC) has large interest in cancer research and emerged as a drug target for cancer therapies (29) and disruption of HKII-mitochondria interaction with peptides corresponding to 15 aa of the N-terminal of HKII disrupt the interaction and triggers apoptosis (38). The mechanism by which this disruption triggers apoptosis remain unknown and we believe assaying HK affinity for G6P-induced inhibition will be most useful to solve this question. HKII binding to mitochondria has also been suggested as key aspect in cardiac protection (10). It has been demonstrated in cardiomyocytes that HK is phosphorylated on Thr^{473} by PKB, a regulatory mechanisms that was found to be associated with translocation of HK to the mitochondria and was associated with reduced affinity for G6P-mediated inhibition (26). This adds an additional regulatory mechanism for control of HK inhibition by G6P, although the presence in intact mature tissue such as skeletal muscle remains to be demonstrated. A simple method to measure HK affinity for G6P-induced inhibition will be a useful tool to address such question.

This is the first assay that takes advantage of 2-DG as substrate to study the sensitivity of HK activity for G6P. Abnormal regulation of HK activity has previously been reported in disease state such as muscle insulin resistance in type 2 diabetes (28) or the increased glycolytic flux present in malignant cancer cells (20). The presented method provides a valuable tool to study the regulation of HK inhibition by G6P under these conditions. Future research could therefore apply this assay in order to clarify the physiological regulation of HK affinity in diseased states such as T2D and cancer.

Funding sources: The study was funded by grants provided by The Lundbeck Foundation (R183-2014-3990/ R180-2014-3887 to JFPW) and The Novo Nordisk Foundation (NNF 16OC0023046, NNF

170OC00272224 to JFPW). Jørgen Jensen received a visiting professorship by Danish Diabetes Academy (DDA) funded by Novo Nordisk.

Acknowledgement: The authors acknowledge the samples from the mouse training study (9) provided by J. Fentz, Section of Molecular Physiology, Department of Nutrition, Exercise and Sports, Faculty of Science, University of Copenhagen.

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Figure legends

Figure 1: Assay reaction by the new established radiochemical filter paper assay that was developed to study hexokinase sensitivity for inhibition by G6P.

A rapid radiochemical filter paper assay for measurement of hexokinase (HK) activity and affinity for G6P was developed. This assay allows for the simple separation of phosphorylated form of the radiolabeled tracer ([^{14}C]- Glucose-phosphate or [^{3}H]-2-Deoxyglucose-phosphate) from the unphosphorylated form ([^{14}C]- Glucose and [^{3}H]-2-deoxyglucose).

In this assay conversion of radiolabeled 2-Deoxyglucose (2-DG) to 2-Deoxyglucose-6-phosphate (2-DG-6-phosphate) is catalyzed by HK. 2-DG-6-phosphate binds to DE81 ion-exchange filter paper (Whatman, Maidstone, UK) and non-phosphorylated 2-DG can be separated by washing the filter paper in excess 60% EtOH. Importantly, 2-DG-6-phosphate does not inhibit HK activity and 2-DG is therefore a perfect substrate for studying the HK sensitivity for G6P. Thus, HK affinity can easily be investigated by performing the radiochemical filter paper assay in the presence of varying G6P concentration.

Figure 2: Comparison of substrates for radiochemical filter paper assay to study hexokinase activity. Hexokinase activity in 6 homogenates derived from a single rat heart was measured in the presence of increasing concentration (0, 0.025, 0.05, 0.1, 0.2, 0.5, 1, 2 and 5 mM) of glucose for ^{14}C-glucose and 2-deoxyglucose (2-DG) for ^{3}H-2-deoxyglucose, respectively (A). Data are representing means and variation (SEM) for 6 homogenates obtained from the same heart tissue. Lineweaver-Burk Plots were used to calculate V_{max} and K_M for ^{3}H-2-DG and ^{14}C-Glucose (B-C) and are given in Table 2.

Figure 3. Sensitivity of the radiochemical filter paper assay

Hexokinase activity in a single rat heart homogenate was measured over time (A) and in the presence of increasing sample/protein concentration for 2 min (B) by use of the radiochemical filter paper assay with 2-DG as substrate and ^{3}H-2-DG as tracer. Product formation of 2-DG-6P was calculated. Each data point represents a triplicate.

The effect of exercise training on HK activity was investigated in mouse muscle from a previous study (9). Quadriceps muscle from sedentary mice and 4-week trained mice (access to running wheel) were compared by the fluorometric and the radiochemical filter paper assay (C). Data from 4 sedentary and 4 trained mice are presented as means ± SEM (n=4). For direct comparison between fresh and frozen tissue, rat soleus muscles were split into two pieces and homogenized as fresh and as frozen tissue respectively (D). HK activity was subsequently determined by fluorometric method and the radiochemical filter paper assay (C). Data represent means ± SEM for 4 soleus muscles that were split into two pieces and investigated by by fluorometric method and the radiochemical filter paper assay (n=4). T-test within fluorometric assay and filter paper assay was used to investigate for the effect of exercise training and fresh/frozen muscle respectively. *** $p<0.001$ for effect of exercise training.

Figure 4. **Hexokinase activity in the presence of increasing 2-DG-6P and G6P concentrations.**

Hexokinase activity in mouse heart was analyzed by the radiochemical filter paper assay with ^{14}C-2-DG as a substrate and assayed in the presence of increasing 2-DG-6P or G6P concentrations (0, 0.1, 0.2, 0.5, 1 mM), respectively. Data represent heart samples from 8 mice and are given as means ± SEM (n=8).

Figure 5. Physiological regulation hexokinase sensitivity for G6P-mediated inhibition. Rat epitrochlearis muscles were isolated and suspended in an *ex vivo* incubation system. Muscles were rested (basal non-stimulated control), stimulated by insulin (10 mU/ml) or forced to contract for 30min. Immediately after incubation, muscles were homogenized and analyzed for HK activity. Sensitivity of HK activity for G6P was analyzed with use radiochemical filter paper assay with ^{14}C-2-DG as a substrate assayed in the presence of increasing G6P concentrations (0, 0.1, 0.2, 0.5, 1 mM).
Data represent values from 8 rested control muscle, 8 insulin-stimulated muscles and 8 electrically contracted muscles and are given as means+/-SEM (n=8).

Table 1

	Flurometric assay	Filter paper assay
Brain	28.76 ± 2.87	26.01 ± 1.31
Heart	10.32 ± 1.04	11.63 ± 1.68
Soleus	2.31 ± 0.05	2.21 ± 0.17
EDL	1.23 ± 0.14	1.14 ± 0.29

Comparison of fluorometric and radiochemical assay for hexokinase activity.
Rat brain, heart, soleus muscle and EDL muscle were homogenized immediately after cervical dislocation. HK activity in homogenate samples was determined by the established fluorometric assay (formation of NADH) and compared to the newly established radiochemical filter paper assay (^{14}C-2-DG-6-phosphate). Activity is given as µmol/g protein/min.
Data present HK activity in different tissues from 8 Wistar rats (n=8) and are presented as means and

the variation among samples is given as SEM (n=8). T-tests revealed no statistical differences between fluorometric and radiochemical assay for measurement of HK activity.

Table 2

	V_{max} (µmol/g protein/min)	K_M (mM)
^{3}H-2DG	**14**	**0.67**
^{14}C-Glucose	**6**	**0.16**

Kinetic parameters for 2-deoxyglucose (2-DG) and Glucose

Lineweaver-Burk Plots were used to calculate V_{max} and K_M for ^{3}H-2-DG and ^{14}C-Glucose (Figure 2B-C) and are given in Table 2.

Figure 1

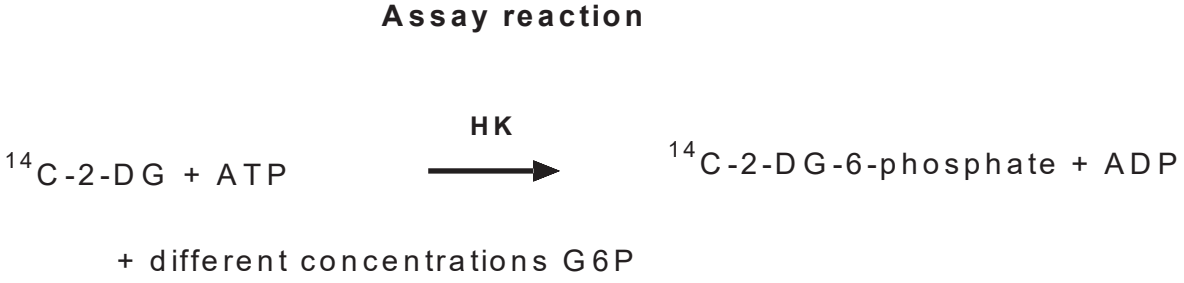
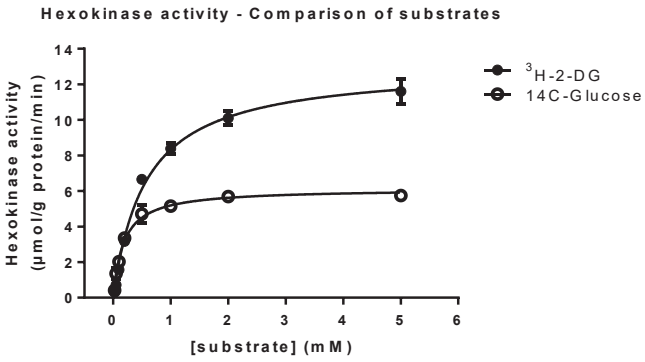
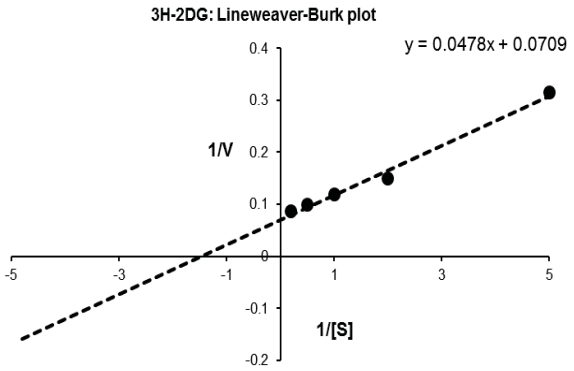

Assay reaction
HK
^{14}C-2-DG + ATP
^{14}C-2-DG-6-phosphate + ADP
+ different concentrations G6P

Figure 2

A


Hexokinase activity - Comparison of substrates
Hexokinase activity (µmol/g protein/min)
[substrate] (mM)
3H-2-DG
14C-Glucose

B


3H-2DG: Lineweaver-Burk plot
y = 0.0478x + 0.0709
1/V
1/[S]

C

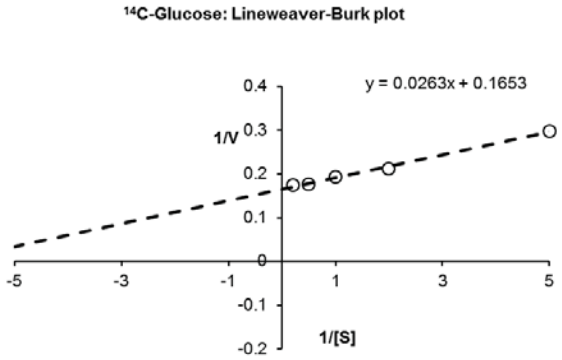
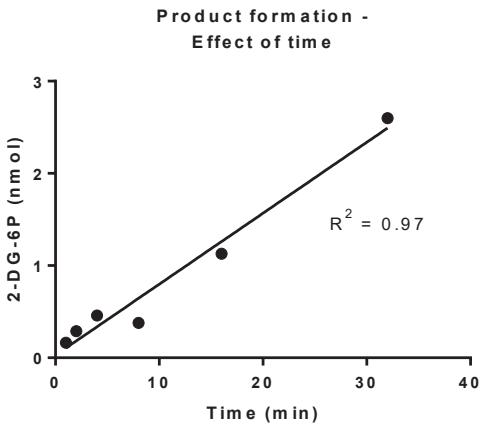
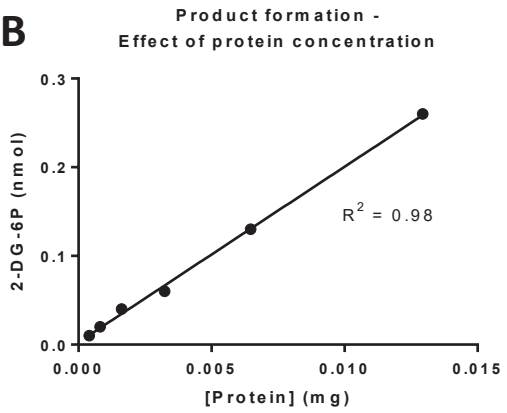

14C-Glucose: Lineweaver-Burk plot
y = 0.0263x + 0.1653
1/V
1/[S]

Figure 3

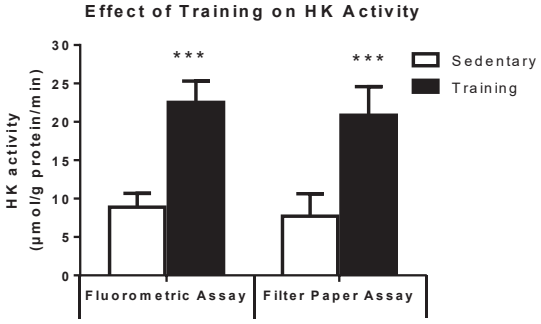
A



B



C



D

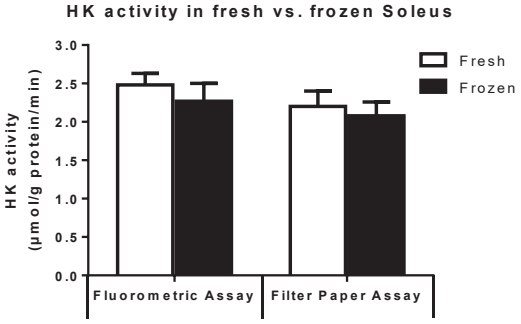


Figure 4

A

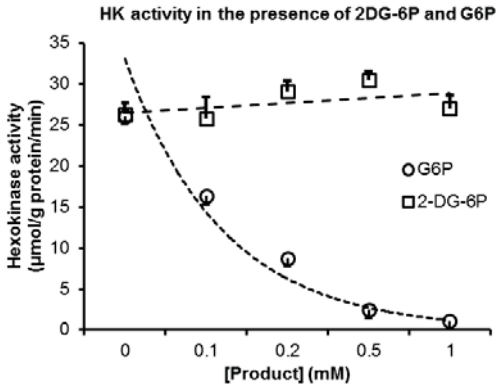
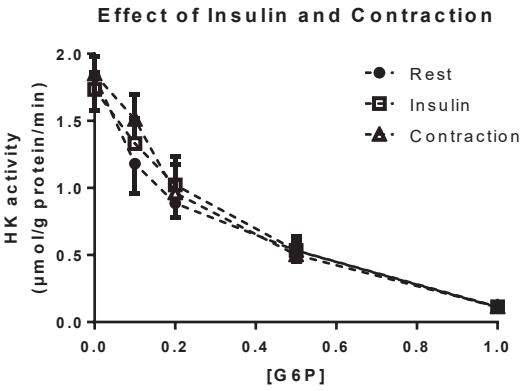

HK activity in the presence of 2DG-6P and G6P
Hexokinase activity (µmol/g protein/min)
35
30
25
20
15
10
5
0
0
0.1
0.2
0.5
1
[Product] (mM)
G6P
2-DG-6P

Figure 5

A


Effect of Insulin and Contraction
HK activity
(µmol/g protein/min)
2.0
1.5
1.0
0.5
0.0
0.0
0.2
0.4
0.6
0.8
1.0
[G6P]
Rest
Insulin
Contraction