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Heidi L. Fritz · Ida W. Smoak · Stacy Branch

Hexokinase I expression and activity in embryonic mouse heart during early and late organogenesis

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Abstract Hexokinase (HK) catalyzes the first step in glucose metabolism, that is, the conversion of glucose to glucose-6-phosphate (G6P). Four HK isoforms have been identified, of which HK-I is predominant in embryonic and fetal tissues. HK-I has been studied in preimplantation embryos and in fetal stages, but little is known about its activity or expression in the early postimplantation embryo. We evaluated HK-I expression, HK-I activity, and glycolytic metabolism in the embryonic mouse heart during early [gestational day (gd) 9.5] and late (gd 13.5) organogenesis. Immunohistochemistry demonstrated that HK-I is localized mainly in the heart at both stages, with stronger expression on gd 13.5. Densitometry after SDS-PAGE/western analysis confirmed higher immunodetectable HK-I protein levels in hearts on gd 13.5 vs gd 9.5. By contrast, RT-PCR demonstrated higher HK-I mRNA expression on gd 9.5 vs gd 13.5. Similarly, cardiac HK-I activity (conversion of glucose to G6P) and glycolysis (conversion of glucose to lactate) were higher on gd 9.5 than on gd 13.5. These results suggest a complex regulation of HK-I expression and activity in the embryonic heart during organogenesis, involving a change in the intrinsic activity of the enzyme with development. HK-I appears to play an important role in glucose metabolism during this critical stage of cardiogenesis.

Introduction

Hexokinase (HK) catalyzes the ATP-dependent conversion of glucose to glucose-6-phosphate (G6P), which is the first step in intracellular glucose metabolism. Four isoforms of HK have been identified, including HK-I, -II, and -III, which have molecular weights (MWs) of approximately 100 kDa and high affinities for glucose, and HK-IV (glucokinase), which has a MW of 50 kDa and a lower affinity for glucose (Katzen 1967). The HK isoforms have considerable amino acid sequence homology, but they represent different gene products and have different distributions in adult tissues. HK-I is ubiquitous and is the major form in brain and erythrocytes. HK-II predominates in insulin-sensitive tissues, including skeletal muscle, heart, and adipose tissue. HK-III is found at low levels in liver, lung, and spleen, and HK-IV is the most abundant form in liver and pancreatic beta cells (Katzen 1967; Ureta 1982).

HK-I is the predominant isoform in embryonic and fetal tissues. HK-I activity was demonstrated in the oocyte (Tsutsumi et al. 1992) and increases significantly by the morula and blastocyst stages (Ayabe et al. 1994; Saito et al. 1994; Johnson et al. 1997). The increase in HK-I activity in the preimplantation embryo appears to regulate the corresponding increase in glucose utilization and glycolysis (Brinster 1968) as the embryo switches from pyruvate to glucose metabolism (Gardner and Leese 1988).

Little is known regarding the expression or activity of HK-I at early postimplantation stages. In the chick embryo, cardiac HK-I activity increases on days 1–4 [comparable to murine gestational days (gd) 8–11.5] followed by constant levels in the heart and increasing levels in the brain (Seltzer and McDougal 1975). Other studies have examined HK expression and activity during fetal and neonatal stages. HK-I was identified by immunohistochemistry in fetal brain, heart, liver, and skeletal muscle on gd 14 in the rat (Coerver et al. 1998), but earlier embryonic stages were not examined. Similarly, HK-I mRNA was found in fetal rat brain, heart, liver, and skeletal muscle at gd 14 (Griffin et al. 1992) and in kidney and lung at gd 18 (Postic et al. 1994), but earlier stages were not examined. HK-I activity is high in guinea pig and rat hearts during fetal stages and declines at birth

H.L. Fritz · I.W. Smoak (✉)
Department of Anatomy, Physiological Sciences, and Radiology, College of Veterinary Medicine, North Carolina State University, 4700 Hillsborough Street, Raleigh, NC 27606 USA
e-mail: ida_smoak@ncsu.edu
Tel.: +1-919-5136322, Fax: +1-919-5136465

S. Branch
Department of Toxicology, North Carolina State University, P.O. Box 7633, Raleigh, NC 27695 USA

(Barrie and Harris 1977; Andres et al. 1984). However, there is no information regarding HK-I expression and activity in the mammalian embryo or embryonic heart during organogenesis, which occurs in the early postimplantation period.

Tissues dependent on glucose for energy typically demonstrate high HK-I activity, as exemplified by transformed cells with increased glycolytic rate and a concomitant increase in HK activity (Bustamente et al. 1981; Nelson and Kabir 1986; Hennipman et al. 1988). The embryonic heart relies on glycolytic metabolism during much of its early development and is highly dependent on glucose as a substrate for energy production and growth (Cox and Gunberg 1972). A shift to include extra-glycolytic energy sources and aerobic (tricarboxylic acid cycle) metabolism occurs during late embryonic and early fetal stages, at approximately gd 12.5–13.5 in the mouse (Cox and Gunberg 1972).

The present study examined the immunohistochemical distribution of HK-I in the mouse embryo at early (gd 9.5) and late (gd 13.5) stages of organogenesis. The heart was found to be a site of strong HK-I expression at both of these stages. The heart was isolated from the embryo and evaluated for levels of HK-I protein and mRNA expression, HK activity, and glycolytic metabolism on gd 9.5 and 13.5. Our results suggest that a complex interaction exists between HK-I expression and activity during organogenesis. This enzyme probably plays an important role in cardiac glucose metabolism during this critical period of heart development.

Materials and methods

Mouse embryos

CD-1 mice were mated overnight and checked for vaginal plugs the following morning, which was considered gd 0.5. Pregnant mice were killed by cervical dislocation on gd 9.5 and 13.5, and embryos were dissected from the uterus and surrounding membranes in cold Tyrode's buffer.

Immunohistochemistry

Whole embryos were isolated on gd 9.5 and 13.5, rinsed well in Tyrode's buffer, and fixed in 4% paraformaldehyde for 24–48 h at 4°C. Embryos were dehydrated in increasing alcohols and xylene and embedded in paraffin. Blocks were sectioned at 5 µm, and sections were floated onto slides and air dried overnight. Sections were then deparaffinized in xylene and rehydrated in decreasing alcohols.

Sections were blocked for endogenous peroxidase activity with 3% H_2O_2 and blocked with 10% non-immune goat serum (BioGenex, San Ramon, Calif., USA), and then incubated at 37°C for 30 min with primary antibody (mouse monoclonal anti-rat HK-I; MAB 1534; Chemicon, Temecula, Calif., USA) diluted 1:100 in 1×phosphate-buffered saline (PBS). Immunoperoxidase staining was performed with a commercially available kit (BioGenex) including biotinylated goat anti-mouse secondary antibody (multilink), peroxidase-conjugated streptavidin (label), and 3-amino-9-ethylcarbazole chromogen–H_2O_2. Slides were counterstained with Mayer's hematoxylin (Zymed, South San Francisco, Calif., USA), coverslipped, and photographed using a Vanox camera. Negative controls were treated with 1×PBS instead of primary antibody.

SDS-PAGE /western analysis

Hearts were isolated from gd 9.5 and 13.5 embryos, rinsed well in Tyrode's buffer, and sonicated in 100 µl cold sonication buffer (50 mM TRIS-HCl, pH 7.4, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 2 µg/µl leupeptin, 2 µg/µl aprotinin). The protein content of each sample was measured with a Bradford (1976) assay using bovine serum albumin (BSA) standards. Aliquots of equal protein (5 µg) were loaded onto a 7.5% Ready Gel (BioRad, Hercules, Calif., USA) and run at 200 V. Proteins were electrophoretically transferred for 1 h at 100 V to a polyvinylidene difluoride membrane (Amersham, Arlington Heights, Ill., USA), which was air dried and stored in a desiccator overnight at 4°C. The membrane was blocked for 1 h in 5% non-fat milk in TTBS [100 ml 10×TRIS-buffered saline (BioRad), 900 ml dH_2O, 500 ml polyoxyethylene-sorbitan monolaurate (Tween 20; Sigma, St. Louis, Mo., USA)], then incubated overnight at 4°C in primary antibody (monoclonal mouse anti-rat HK-I; MAB 1534; Chemicon) diluted 1:1000 in TTBS. The specificity of the HK-I band was demonstrated by exposing test lanes to TTBS without primary antibody. The membrane was incubated for 1 h in secondary antibody (goat anti-mouse; Amersham) diluted 1:10,000 in TTBS. Chemiluminescent signal (ECL-Plus; Amersham) was detected by exposure to X-ray film, and HK-I bands were confirmed by comparison with MW markers. Films were scanned into Adobe PhotoShop, and band densities were calculated as volume percent using IP LabGel (Signal Analytics, Vienna, Va., USA) software.

Reverse transcriptase–polymerase chain reaction

Embryos were dissected free of maternal tissues on gd 9.5 and 13.5 in cold diethyl pyrocarbonate-treated PBS. Hearts were isolated, pooled, and snap frozen in liquid nitrogen and stored at –70°C until assayed. Total RNA was extracted from pooled embryonic hearts using the Rneasy kit (Qiagen, Santa Clarita, Calif., USA). The preamplification kit (Gibco-BRL, Gaithersburg, Md., USA) was used for reverse transcription of RNA to DNA. An oligo (dT) primer was used for cDNA synthesis and the cDNA product used as the template for PCR reactions, using positive and negative controls throughout. A master mix approach decreased sources of variation between PCR reactions. Cyclophilin was used as an internal standard. Primers for HK-I and cyclophilin were designed from mRNA sequences obtained from GenBank. The PCR products were analyzed on a 1.6% agarose gel and quantified by densitometry (IP LabGel), and results were expressed as density ratios of HK-I mRNA to cyclophilin mRNA.

Hexokinase activity

Hearts were isolated and pooled from gd 9.5 embryos (20–25 hearts) and gd 13.5 embryos (2–3 hearts), rinsed well in Tyrode's buffer, and sonicated in 100 µl cold sonication buffer (10 mM TRIS-HCl, pH 7.4). HK-I activity was evaluated using a modification of previously described methods (Gots and Bessman 1973) to measure conversion of glucose to G6P. Protein content in each sample was measured with a Bradford (1976) assay using BSA standards. Heart samples were suspended in sonication buffer at a protein concentration of 500 µg/ml, and 100 µl (50 µg) was added to the reaction mixture (107 mM TRIS-HCl, pH 7.4, 32.2 mM $MgCl_2$, 1.6 mM glucose, 5.36 mM ATP) and substrate (1 µCi [U]-^{14}C-glucose) and allowed to incubate for 10 min at 25°C. The reaction was terminated by adding 4 ml of stop solution (1 M glucose, 170 mM NH_4OH). The product was adsorbed by adding 250 mg Dowex 2×8–100 (Sigma) and agitating 4 times. The supernatant was discarded and the Dowex was washed 4 times with 4 ml 170 mM NaOH. The product was eluted by adding 1 ml 1 N HCl and agitating 4 times. A 500-µl aliquot of supernatant was added to scintillation cocktail and assayed for ^{14}C. Control vials without heart sample, substrate, or both, were run in

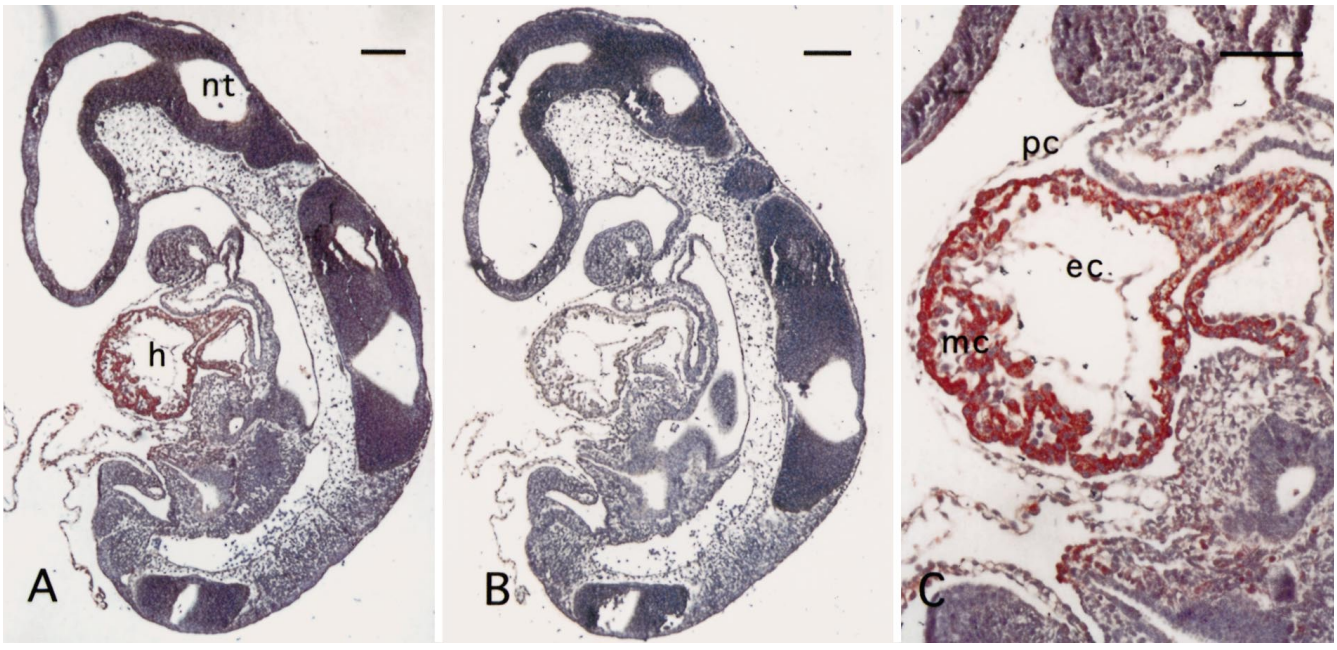

Fig. 1A–C Immunohistochemical demonstration of hexokinase (HK)-I in gestational day (gd) 9.5 mouse embryos. Sections were reacted with anti-HK-I antibody or phosphate-buffered saline (PBS) control and counterstained with hematoxylin. Regions of HK-I immunoreactivity are *red*. **A** Section of embryo incubated with anti-HK-I antibody, demonstrating HK-I localization in the heart (*h*) and neural tube (*nt*). **B** Adjacent section of embryo exposed to PBS, demonstrating no immunoreactivity. **C** Magnified view of heart, demonstrating strong HK-I staining in myocardium (*mc*) and light HK-I staining in pericardium (*pc*) and endocardium (*ec*). *Bar* 0.1 mm

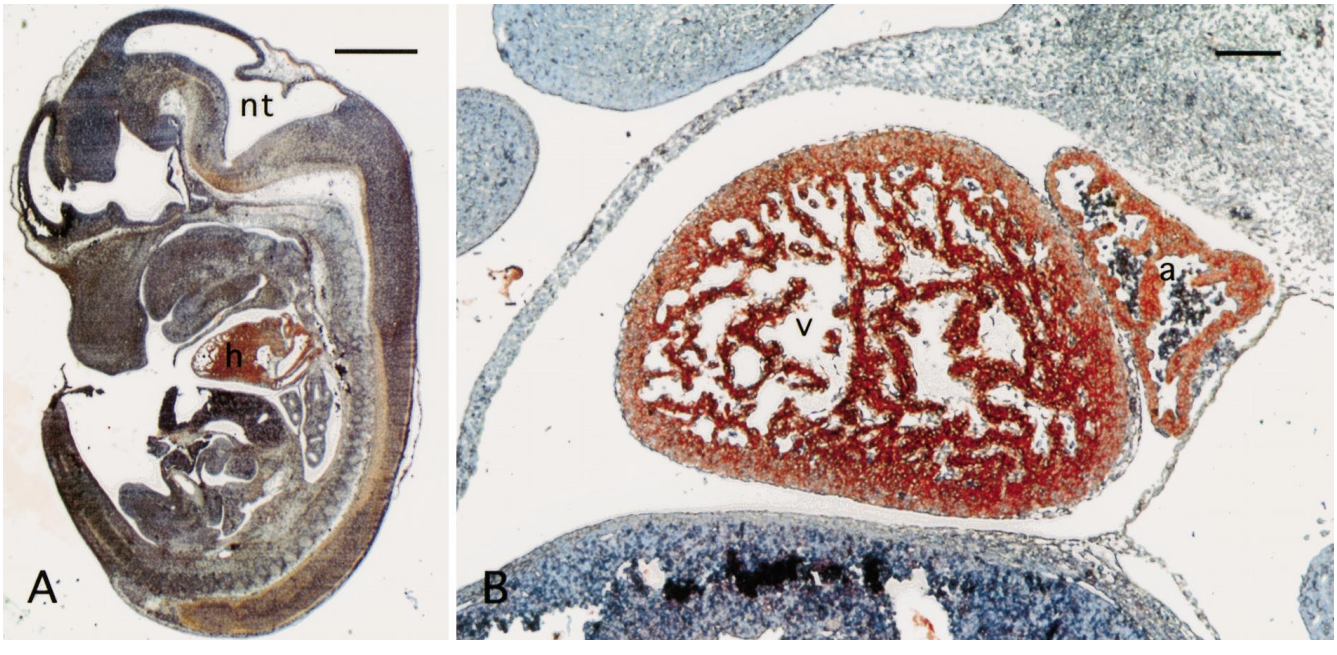

Fig. 2A,B Immunohistochemical demonstration of HK-I in gd 13.5 mouse embryos. Sections were reacted with anti-HK-I antibody and counterstained with hematoxylin. Regions of HK-I immunoreactivity are *red*. **A** Section of embryo demonstrating HK-I localization in the heart (*h*) and neural tube (*nt*). *Bar* 1 mm. **B** Magnified view of heart, demonstrating intense HK-I staining in the atrial (*a*) and ventricular (*v*) myocardium. *Bar* 0.1 mm

parallel, and the radioactivity in these samples was subtracted from test samples to determine the amount of ^{14}C-G6P produced. Results were expressed as nmol G6P produced per g protein of heart tissue.

Glycolysis

Hearts were isolated from gd 9.5 and 13.5 embryos and placed individually in 10-ml vials containing 1 ml culture medium (75% rat

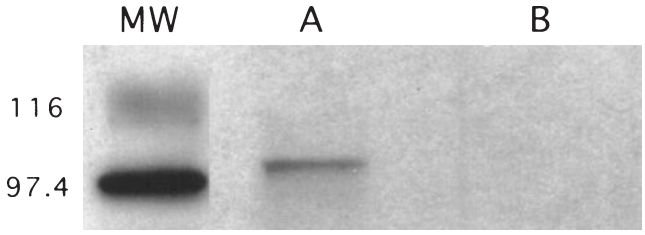

Fig. 3A,B SDS-PAGE/western analysis of gd 13.5 heart homogenate demonstrating specificity of HK-I antibody. HK-I band appears at approximately 100 kDa, between molecular weight (MW) markers 97.4 and 116 kDa. **A** Gel lane exposed to anti-HK-I antibody. **B** Gel lane exposed to TTBS (100 ml 10×TRIS-buffered saline, 900 ml dH_2O, 500 ml Tween 20) control

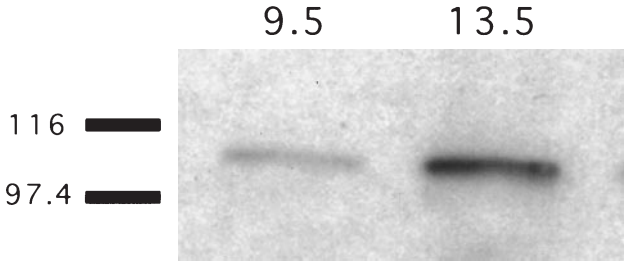

Fig. 4 HK-I band produced by SDS-PAGE/western analysis in gd 9.5 and gd 13.5 mouse hearts. HK-I band is at approximately 100 kDa, between MW markers 97.4 and 116 kDa, and appears more dense at gd 13.5 than at gd 9.5

serum, 25% Tyrode's buffer) and 1 µCi [U]-^{14}C-glucose. Incubation was for 1 h on a rotating wheel in a 37°C incubator. Lactate produced by each heart was separated using a modification of previously described methods (Barker and Summerson 1941), in which 1 ml medium was deproteinated with 1 ml 20% trichloroacetic acid and centrifuged. A 1-ml aliquot of supernatant was combined with 1 ml copper sulfate, 8 ml water, and 1 g calcium hydroxide, and the solution was vortexed and centrifuged. A 500-µl aliquot of supernatant was added to scintillation cocktail and assayed for ^{14}C. A control vial without an embryonic heart was run in parallel, and the radioactivity in this sample was subtracted from test samples to determine the amount of ^{14}C-lactate produced. Results were expressed as µmol lactate produced per g protein of heart tissue.

Statistical analysis

Numerical data were analyzed with an analysis of variance for overall significance and a Tukey's test for between-group comparisons. SAS software (SAS Institute, Cary, N.C., USA) was used for all analyses, with a significance level of $P<0.05$ throughout.

Results

Immunohistochemistry

The embryonic heart undergoes significant morphological changes between gd 9.5 and 13.5, including an approximate threefold increase in overall dimensions and an increase in thickness and complexity of the myocardium (Figs. 1C, 2B). Immunohistochemistry demonstrated HK-I staining in the gd 9.5 embryo that was localized in the heart and neural tube (Fig. 1A). Specificity of the HK-I antibody was demonstrated by a lack of staining in parallel sections not treated with primary antibody (Fig. 1B). Within the gd 9.5 heart, HK-I is predominantly localized in the myocardium, with light staining in the pericardium and endocardium (Fig. 1C). HK-I is more diffusely distributed throughout the embryo on gd 13.5, with intense staining in the heart and light staining in the neural tube (Fig. 2A). Within the gd 13.5 heart, HK-I is predominantly localized in the myocardium, with little difference in staining intensity between atrium and ventricle (Fig. 2B). There is no HK-I staining in the endocardial cushions (not shown). Sections of gd 9.5 and 13.5 embryos processed in parallel for HK-I by immunohistochemistry consistently demonstrated greater intensity of cardiac HK-I on gd 13.5 than on gd 9.5 (Figs. 1C, 2B).

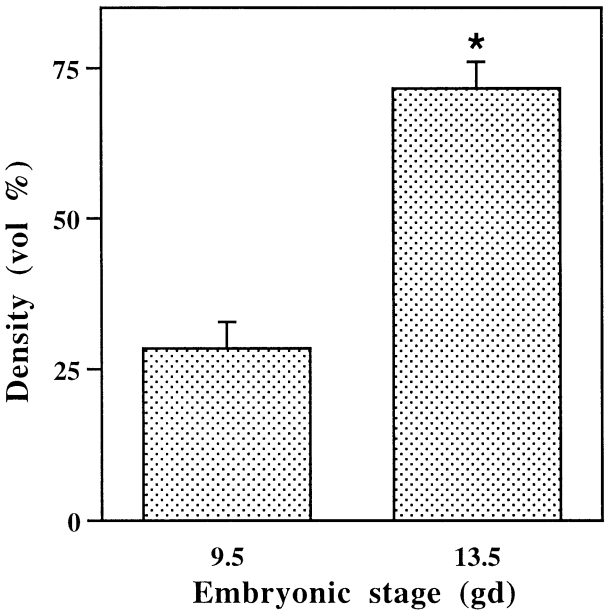

Fig. 5 Density of bands produced by SDS-PAGE/western analysis of HK-I in gd 9.5 and gd 13.5 mouse hearts. *Bar heights* represent mean volume percent density + SEM of HK-I bands from six gels. *Asterisk* $P<0.0001$

SDS-PAGE/western analysis

HK-I appeared as a specific band at approximately 100 kDa in gd 13.5 embryonic heart homogenates evaluated by SDS-PAGE/western analysis (Fig. 3A). Specificity of the HK-I antibody was demonstrated by the absence of the band in negative control lanes that were not treated with primary antibody (Fig. 3B). The density of the HK-I band in gd 13.5 heart samples appeared consistently greater than that of gd 9.5 heart samples (Fig. 4). This impression was confirmed by densitometry, which demonstrated a significant difference in cardiac HK-I levels at these two stages. Mean band density was almost

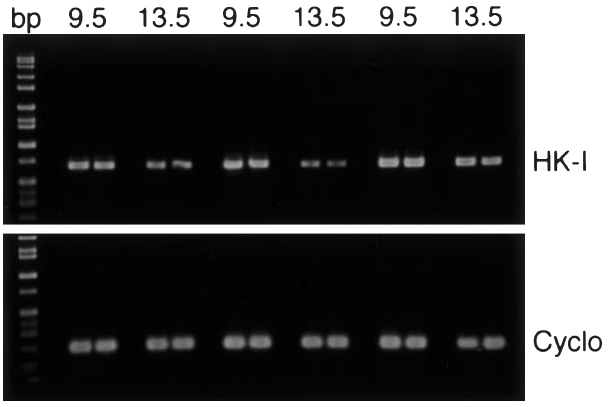

Fig. 6 RT-PCR agarose gel demonstrating mRNA bands for HK-I (681 bp *top row*) and cyclophilin (212 bp *bottom row*) from three sets of gd 9.5 and gd 13.5 heart homogenates

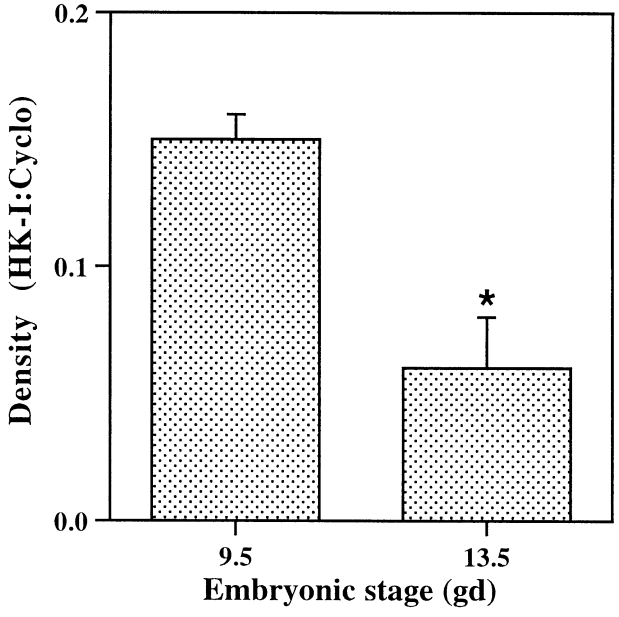

Fig. 7 Density of HK-I mRNA bands produced by RT-PCR on gd 9.5 and gd 13.5 heart homogenates. *Bar heights* represent ratio of HK-I:cyclophilin band density from three sets of samples. *Asterisk* $P<0.013$

threefold higher in gd 13.5 hearts than in gd 9.5 hearts, based on an average of six gels (Fig. 5).

Reverse transcriptase–polymerase chain reaction

Our RT-PCR results demonstrate higher HK-I mRNA expression in gd 9.5 hearts than in gd 13.5 hearts (Fig. 6). Band densities, calculated as ratios to the internal standard cyclophilin, are approximately threefold higher in gd 9.5 vs gd 13.5 hearts, based on the average of three pairs of samples (Fig. 7).

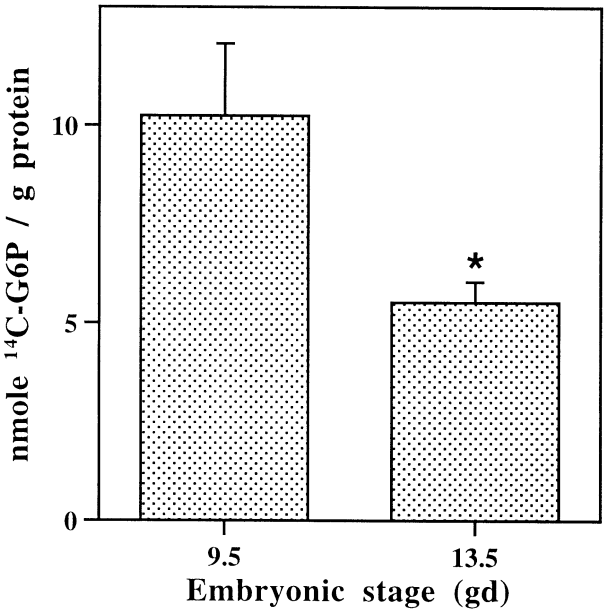

Fig. 8 HK activity in gd 9.5 and gd 13.5 mouse hearts determined by conversion of [U]-^{14}C-glucose to ^{14}C-glucose-6-phosphate in 10 min. *Bar heights* represent mean + SEM of eight assays per stage performed on pooled heart samples. *Asterisk* $P<0.027$

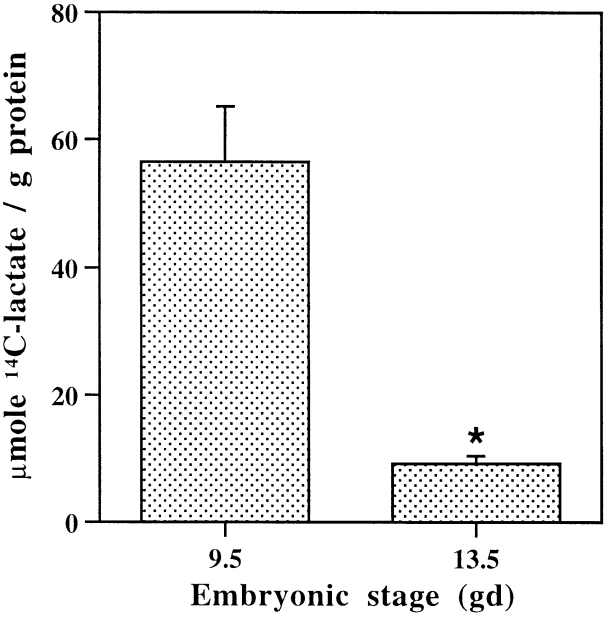

Fig. 9 Glycolysis in gd 9.5 and gd 13.5 mouse hearts determined by conversion of [U]-^{14}C-glucose to ^{14}C-lactate in 1 h. *Bar heights* represent mean + SEM of 16 assays per stage performed on individual hearts. *Asterisk* $p<0.0001$

Hexokinase activity

The activity of hexokinase, as measured by the conversion of [U]-^{14}C-glucose to ^{14}C-G6P in 10 min, was significantly (approximately twofold) higher per g protein in gd 9.5 hearts than in gd 13.5 hearts (Fig. 8). Results represent the average of eight assays per stage performed on pooled heart samples.

Glycolysis

Glycolytic metabolism, as measured by the production of ^{14}C-lactate from [U]-^{14}C-glucose in 1 h, was significantly (more than sixfold) higher per g protein in gd 9.5 hearts than in gd 13.5 hearts (Fig. 9). Results represent the average of 16 assays per stage performed on individual hearts.

Discussion

Hexokinase is a critical enzyme in glucose metabolism and catalyzes the phosphorylation of glucose to G6P for progression through glycolysis and other metabolic pathways. Four HK isoforms have been identified and are differentially distributed according to tissue type and stage of development. HK-I is ubiquitous in adult tissues and is the most predominant isoform in embryonic and fetal stages. Previous work has demonstrated HK-I activity in the embryonic chick heart and in rodent hearts during fetal (gd 14 and older) and neonatal stages. This study represents the first to examine HK-I activity and expression as they relate to glucose metabolism in the organogenesis-stage rodent embryo.

Our immunohistochemistry studies examined the distribution of HK-I in mouse embryos during early (gd 9.5) and late (gd 13.5) organogenesis. At both stages, the heart was a primary site of HK-I expression, suggesting an important role for this enzyme in the organogenesis-stage heart. HK-I expression was localized mainly in myocardial cells at both stages and appeared equally intense in atrial and ventricular myocardium. Interestingly, the intensity of immunohistochemical staining for HK-I appeared consistently stronger in the gd 13.5 heart than in the gd 9.5 heart.

Results of SDS-PAGE/western analysis confirmed the apparent differences in HK-I expression produced by immunohistochemistry. Densitometric analysis demonstrated almost threefold higher HK-I expression in hearts on gd 13.5 than on gd 9.5, normalized by equal protein loaded per gel lane. Thus, the expression of immunodetectable HK-I protein in the embryonic heart increases between early and late organogenesis. Previous work has demonstrated high and fairly constant HK-I levels in the heart throughout fetal stages (Griffin et al. 1992; Coerver et al. 1998), suggesting the importance of this enzyme for glycolytic metabolism in the developing heart.

Despite an increase in cardiac HK-I protein between gd 9.5 and 13.5, HK-I mRNA expression, as determined by RT-PCR, is approximately threefold higher in gd 9.5 vs gd 13.5 hearts. In addition, enzymatic activity of HK, as measured by the conversion of glucose to G6P, is approximately twofold higher per g protein in gd 9.5 hearts than in gd 13.5 hearts. Similarly, the glycolytic rate, as measured by the conversion of glucose to lactate, is significantly (more than sixfold) higher per g protein in gd 9.5 hearts than in gd 13.5 hearts. These results are consistent with previous findings that the embryonic heart is dependent on glucose and glycolytic metabolism at early stages of organogenesis and begins to use extra-glycolytic energy sources during late embryonic and early fetal stages (Cox and Gunberg 1972). The mismatch between HK-I protein and mRNA expression is not understood but suggests a complex developmental regulation of HK-I expression and activity in the embryonic heart. Our results suggest that HK-I has higher intrinsic activity and mRNA expression, despite lower enzyme expression, in the embryonic heart at early vs late stages of organogenesis.

In summary, the embryonic heart is a site of strong and increasing expression of HK-I protein between early (gd 9.5) and late (gd 13.5) stages of organogenesis. However, HK-I mRNA expression and enzymatic activity and glycolytic metabolism in the heart decline during this period, suggesting a change in intrinsic activity of the enzyme. The regulation of this phenomenon is not understood. Our results further suggest that HK-I plays a critical role in glucose metabolism within the embryonic heart during organogenesis.

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