ABSTRACT

GHATNEKAR, GAUTAM SUDHIR. Hypoglycemia-induced effects on cell proliferation, cell death, and glucose metabolism in organogenesis-stage embryonic mouse heart. (Under the direction of Dr. Robert Anholt and Dr. David Malarkey).

Hypoglycemia is a common clinical condition and is believed to cause cardiac malformations in offspring born to diabetic mothers. This study investigates the in vivo and in vitro effects of hypoglycemia on glucose metabolism, cell death and cell proliferation in the organogenesis-stage embryonic mouse heart. This study also offers a possible way to prevent excessive cell death due to hypoglycemia by over expressing Hsp70. Effects on glucose metabolism were evaluated by 13C-NMR spectroscopy to study glucose utilization and lactate production in embryonic day (E) 10.5 embryonic hearts exposed to hypoglycemia (40 mg/dl glucose) compared to normoglycemia (150 mg/dl glucose). Cell death was evaluated by TUNEL, Lysotracker®, active caspase-3 protein expression in vivo and in vitro, and by flow cytometry using TUNEL and myosin heavy chain in vivo. Cell proliferation was evaluated by p53, and PCNA immunohistochemistry and Western analysis. Hsp70 over-expression was achieved in isolated heart cultures and the hearts post-culture were stained with Lysotracker® or used for Western analysis to analyze Hsp70 over-expression and active caspase-3 expression. 13C-NMR spectroscopy revealed increased lactate production in hearts exposed to hypoglycemia as compared to
normoglycemia indicative of increased glucose metabolism by glycolysis. TUNEL, Lysotracker®, and active caspase-3 assays indicate increased cell death induced by hypoglycemia in E9.5 and E10.5 embryonic hearts but not in E8.5 embryonic hearts. Flow cytometry results are similar to TUNEL and Lysotracker® results. p53 and PCNA analysis demonstrates decreased cell proliferation in response to hypoglycemia during organogenesis. Over-expressing Hsp70 helped prevent excessive cell death induced by hypoglycemia in embryonic mouse hearts. Over-expressing Hsp70 significantly reduced active caspase-3 expression as demonstrated by Western analysis. The results indicate that 24 hr hypoglycemic exposure in vivo and in vitro significantly alters glucose metabolism, cell death and cell proliferation in the organogenesis-stage embryonic mouse heart. These effects may eventually contribute to lethality in the embryo or congenital cardiac defects observed in diabetic offspring.
Hypoglycemia-induced effects on cell proliferation, cell death, and glucose metabolism in organogenesis-stage embryonic mouse heart

by

GAUTAM SUDHIR GHATNEKAR

A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

COMPARATIVE BIOMEDICAL SCIENCES

RALEIGH

2004

APPROVED BY

Dr. Gregory J. Cole

Dr. Peter Farin

Dr. David Malarkey

(Chair of Advisory Committee)

Dr. Robert Anholt

(Chair of Advisory Committee)
DEDICATION

I dedicate my thesis to God and my parents Dr. Sudhir D. Ghatnekar and Mrs. Anjali S. Ghatnekar, without whom this journey would have been impossible. My parents and my family have given me endless support, love, and encouragement, helped me learn from my mistakes, and continue towards my goal. To all my friends who believed in me and who stood by me through tough times.
BIOGRAPHY

Gautam Sudhir Ghatnekar was born in Mumbai, India. He attended school and college in Mumbai, formerly called Bombay. During his school years he recognized his passion for Veterinary Medicine. He completed his Bachelor of Veterinary Science degree from Bombay veterinary College in December 1998. Gautam practiced Veterinary Medicine in Mumbai for 2 years and then decided to join the Comparative Biomedical Sciences program at North Carolina State University, Raleigh, North Carolina in fall 2000.

At North Carolina State University, College of Veterinary Medicine, Gautam taught gross anatomy for 2 semesters to first year DVM students. He has been very active and involved in the program. In 2001 he was elected as the student representative on the Comparative Biomedical Sciences Graduate Studies Committee. In 2002 he was elected as the President of College of Veterinary Medicine Graduate Student Association. He started a travel award for graduate students at the College of Veterinary Medicine, encouraging them to attend and present their research at conferences.

Gautam received several awards during the course of his graduate work, one of the most prestigious been the Marie W. Taubeneck award given by the Teratology Society to one graduate student every year for excellence in research and leadership offered to fellow students. His Ph.D. work at North Carolina State University is in Cell and Developmental Biology with emphasis in Teratology.
ACKNOWLEDGEMENTS

I want to thank Dr. Robert Anholt, the chair of my committee for his exceptional support and mentoring, which has enabled me to successfully complete my Ph.D. work at N. C. State University.

I would also like to thank Dr. David Malarkey, the co-chair of my committee and Dr. Peter Farin, my minor representative on the committee for their guidance and support. They were generous with their time, knowledge and experience.

A very special thanks Dr. Gregory Cole, my committee member. I don’t have words to describe his mentoring, support, kindness, friendship and educational input in my research. Without Dr. Cole I would not have been able to complete this journey successfully.

I fondly thank my colleagues and friends at N. C. State, particularly Nia Joyner, Dr. Francisco Javier Cisneros, Regina Leonard, Dominique Williams, Dr. Rajagopal Sriperumbudur, and Dr. Min Jung Kim for their honest friendship and support for all these years.

I sincerely want to thank Dr. Ju-Ahng Lee for his interest and contribution towards my project. Ju-Ahng and I have spent many a sleepless nights together in the lab, doing science and talking basketball. I consider myself really lucky to have a friend and mentor like him.
I have a special thank you for Dr. Rhonda Sutton for keeping my hope alive. Her words of wisdom have always acted as fuel to keep my learning machine functional.

I want to particularly thank Dr. Robert Sowell, Dean of Graduate School, and Dr. Neil Olson, Associate Dean of Vet. School for supporting me and helping me stay the course.

I want to thank the administrative staff in the Dept. of Molecular Biomedical Sciences, particularly Becky, Sue, Vicki, Marj and Jennifer. They have been really supportive through the last 4 years.

I want to thank Kristina Coxe. I am really grateful for her honest friendship, support, and assistance through the years.

Very special thanks to my dad, mom, my younger brother, and Granny. Their emotional and spiritual support has been great. I have really appreciated the frequent visits by my mother all the way from India to take care of me and to get me through tough times. I would have never made it without my family by my side.

I also want to thank Dr. Jodi Gookin for her friendship, advice, trust, and support.

I want to acknowledge Dr. Ida Smoak since most of my work was done in her laboratory.

I really appreciate the support by National Institute of Health and American Heart Association for funding my research.
Last but not the least I want to thank all the rats and mice who have sacrificed themselves for the cause of scientific research. I hope I have done some justice to their sacrifice.
TABLE OF CONTENTS

List of Tables...........................................................................................................X

List of Figures............................................................................................................XI

Introduction..............................................................................................................1
  Heart Development.................................................................................................3
  Cell Proliferation in Embryonic Heart.................................................................6
  Cell Death in Embryonic Heart.............................................................................6
  p53 in the Embryonic Heart..................................................................................12
  Caspase-3 in the Embryonic Heart.......................................................................13
  Hsp70 in the Embryonic Heart.............................................................................15
  Research Overview...............................................................................................17

Chapter 1

$^{13}$C-NMR study of hypoglycemia-induced glycolytic changes in embryonic mouse heart.................................................................18

Abstract..................................................................................................................18

Introduction............................................................................................................20

Methods.................................................................................................................23
Chapter 2

Hypoglycemia Induced Changes in Cell Death and Cell Proliferation in Organogenesis Stage Embryonic Mouse Heart with Emphasis on E9.5

Abstract
Introduction
Materials and Methods
Results
Discussion

Chapter 3

Anti-Apoptotic Effects of hsp70 In Cultured Embryonic Mouse Hearts

Abstract
Introduction
Materials and Methods
Results
Discussion........................................................................................................134

General Discussion.............................................................................................138

Literature Cited....................................................................................................147
LIST OF TABLES

Table 1. Different types of cell death.................................................................8

Table 2. $^{13}$C-NMR parameters used in 13C-NMR experiments.......................27

Table 3. Resorption data from pregnant mice exposed to hypoglycemia or control conditions on E9.5 for 24 hr and sacrificed on E18.5..............................65
LIST OF FIGURES

Figure 1. Embryonic heart development during organogenesis ..................5
Figure 2. General overview of the apoptotic pathway in mammalian cell.........11
Figure 3. $^{13}$C-NMR spectra for $^{13}$C enriched normoglycemic medium..........30
Figure 4. $^{13}$C-NMR spectra for $^{13}$C enriched hypoglycemic medium..........33
Figure 5. Average ratios + S.E.M. of post-culture [3-$^{13}$C-lactate] to pre-culture [1-$^{13}$C-glucose] .................................................................35
Figure 6. Schematic of glycolysis demonstrating disposition of $^{13}$C atoms from metabolism of U-$^{13}$C-glucose to $^{13}$C-lactate .................................37
Figure 7. Morphological effects of hypoglycemia on E8.5 .........................61
Figure 8. Morphological effects of hypoglycemia on E9.5 .........................62
Figure 9. Morphological effects of hypoglycemia on E10.5 .......................63
Figure 10. Bar graph demonstrating significant decrease in mean total cardiac protein in hearts of E9.5 embryos exposed to hypoglycemia .................64
Figure 11. Graph depicting the mean serum glucose levels during a 24 hr treatment period .................................................................66
Figure 12. Uterus from a mouse exposed to 24 hr hypoglycemia in vivo on E9.5 and sacrificed on E 18.5 .........................................................67
Figure 13(A-C). TUNEL staining in E8.5 embryonic hearts .......................71
Figure 13(D-E). Lysotracker staining in E8.5 embryonic hearts .................73
Figure 14(A-F). TUNEL, CardioTACS, and Lysotracker staining in E9.5 embryonic hearts……………………………………………………………………………………………………….75

Figure 14G. Quantitation of apoptotic cells in E9.5 hearts analyzed by TUNEL assay…………………………………………………………………………………………………………………77

Figure 14H. Quantitation of apoptotic cells in E9.5 hearts analyzed by Lysotracker assay…………………………………………………………………………………………………………………78

Figure 15(A-C). TUNEL staining in E10.5 embryonic hearts………………………………79

Figure 15(D-F). Lysotracker staining in E10.5 embryonic hearts………………………….81

Figure 16. Flow cytometry on cardiac cells from embryos exposed in vivo on E9.5 to 24-hr hypoglycemia compared to controls……………………………………………………………..84

Figure 17. Immunohistochemistry of active caspase-3 subunit in E8.5 embryonic hearts……………………………………………………………………………………………………………89

Figure 18. Immunohistochemistry and Western analysis for active caspase-3, total p53 and phosphorylated p53 in E9.5 embryonic hearts……………………………………………………………..90

Figure 19. Immunohistochemistry of active caspase-3 subunit in E10.5 embryonic hearts……………………………………………………………………………………………………………92

Figure 20. Immunohistochemistry of total p53 in E8.5 embryonic hearts……………..93

Figure 21. Immunohistochemistry of total p53 in E10.5 embryonic hearts……………..94

Figure 22. Electron microscopy pictures showing mitochondrial changes……………..96

Figure 23. Immunohistochemistry and Western analysis of PCNA in E9.5 embryonic hearts……………………………………………………………………………………………………….97

Figure 24. Immunohistochemistry for Hsp70 in E9.5 embryonic hearts………………..122
Figure 25. Transfection of primary cardiac cell culture with pCMV-hsp70-hrGFP over expression vector.................................................................123

Figure 26. Isolated hearts stained with Lysotracker assay.............................126

Figure 27(A-C). Western analysis for Hsp70 and active caspase-3................129

Figure 27D. Quantitation of Hsp70 Western analysis................................131

Figure 27E. Quantitation of Active caspase-3 Western analysis....................132
INTRODUCTION

Congenital heart malformations contribute significantly to birth defects in the human population and are responsible for a large number of neonatal deaths. The incidence of birth defects in babies of diabetic women appears two to three times higher than in normal population. According to the American Diabetes Association approximately 9.3 million or 8.7% of all women over age 20 in the United States have diabetes. However, about one-third of them do not know it. The prevalence of diabetes is at least 2-4 times higher among African American, Hispanic/Latino, American Indian, and Asian/Pacific Islander women than among white women. Due to increasing lifespan of women and the rapid growth of minority populations, the number of women in the United States at high risk for diabetes and its complications is increasing.

An adequate supply of glucose to the embryonic heart during organogenesis is critical to normal development of cardiac function and metabolism (Smoak & Sadler, 1990; Smoak & Sadler, 1991; Smoak, 1997). The embryonic heart demonstrates a dependence on glucose and glycolytic metabolism. Glucose is necessary to maintain maximal heart rate under anaerobic conditions until embryonic day (E) 11 in rat embryos, followed by a shift to include extra-glycolytic energy sources and aerobic metabolism at E13
(Cox & Gunberg, 1972a). The embryonic heart appears to be particularly sensitive to alterations in glucose availability, which may contribute to cardiac dysmorphogenesis and abnormal cardiac function. For women with diabetes, excellent blood glucose control before conception and then throughout pregnancy is vital to the health of the baby and the mother. Statistics presented by the American Diabetes Association reveal that the rate of major congenital malformations in babies born to women with preexisting diabetes varies from 0-5% among women who receive preconception care to 10% among women who do not receive preconception care.

Hypoglycemia is a commonly occurring clinical condition in humans, seen as a side effect of diabetes mellitus therapy (Nilsson et al., 1988), and as a result of disease states such as starvation and tumors (Steding & Seidi, 1981; Senior & Sadeghi-Nejad, 1989). Hypoglycemia interferes with normal cardiac development and function, since glucose is the only source of energy production and growth in early organogenesis (Cox & Gunberg, 1972b). In vitro exposures to hypoglycemia have resulted in cardiac malformations in laboratory animals, even after brief periods of exposure, such as ≥ 2 hr (Buchanan et al., 1986; Smoak & Sadler, 1990; Smoak, 1997). E9.5 embryonic hearts demonstrated an increase in cardiac glycolysis after exposure to 60 mg/dl glucose in vitro for 4 hr (Peet & Sadler, 1996). Increased incidence of congenital malformations has been found
in the offspring of both human and experimental diabetic pregnancies (Kitzmiller et al., 1978; Eriksson et al., 1991). Alterations in neural closure, central nervous system defects and cardiac malformations are the most common congenital malformations in children from diabetic women (Martinez-Frias, 1994) and in insulin dependent rat models of diabetes (Eriksson et al., 1991). The malformations seen include an overall decrease in size, decrease in contractile rate, and depending on the extent of insult, deformities such as Interventricular septal defect, Pulmonic stenosis, Interatrial septal defects and more (Steding & Seidi, 1981).

**Heart Development**

The first stage in mammalian cardiogenesis is the formation of the cardiogenic plate. Subsequently, the left and right cardiogenic primordia come together to form a single median heart tube (endocardial tube). Myocardium is formed from the mesodermal layer surrounding the heart tube. Between the myocardial and endocardial layers is a layer of loose connective tissue, called the cardiac jelly. The third stage of cardiogenesis is the formation of the cardiac loop. Finally, there is atrial and ventricular septation, separation of the outflow tracts, and development of valves (Steding & Seidi, 1980). The period of fusion, folding and septation is described as the critical period of cardiogenesis (Noden & DeLahunta, 1985; Smoak, 2000) (Fig 1). The most active period of cardiac
development occurs between E9 and E10.5 in the murine embryo, which is equivalent to E21 to E30 in the human embryo (Smoak, 1997). This is the stage when the embryo develops from the 10-somite stage (cardiac contractions and heart looping) to the 30-somite stage (septation and valvulogenesis). In this critical stage of development, brief (2 - 4 hr) exposures to hypoglycemia alter the embryonic heart morphologically, functionally as well as metabolically (Peet & Sadler, 1996; Smoak, 1997; Ghatnekar et al., 2002; Ghatnekar et al., 2004). Cardiac morphogenesis is regulated by coordination of cell transformation, cell proliferation and cell death.
Figure 1. Embryonic heart development during organogenesis in a mouse embryo. (Noden and DeLahunta, 1985)

A. Embryonic heart around 10-somite stage. This corresponds to E8.5 in mouse embryonic development.

B. Embryonic heart around 20-somite stage. This corresponds to E9.5 in mouse embryonic development.

C. Embryonic heart around 30-somite stage. This corresponds to E10.5 in mouse embryonic development.
Cell Proliferation in the Embryonic Heart

Cell proliferation is also an important developmental process. The rate of cell division is a tightly regulated process that is intimately associated with growth, differentiation and tissue turnover.

PCNA, or proliferating cell nuclear antigen, is commonly used to measure and detect changes in cell proliferation. PCNA is expressed only in proliferating cells and thus can be used to selectively mark proliferating cells in cell populations and tissues. PCNA can be used to demonstrate levels of cellular proliferation in Western analysis and tissue distribution of cell proliferation using immunohistochemistry.

Cell proliferation studies using PCNA in day 4 to day 8-chick embryonic heart showed occurrence of cell proliferation throughout the embryonic heart, including the myocardium (Keyes & Sanders, 1999). PCNA is a good marker for observation of proliferative activity in developing tissues (Kaminski et al., 1999), and in developing and adult cardiac cells (Quaini et al., 1994; Marino et al., 1996).

Cell Death in the Embryonic Heart

The study of cell death is now the focus of both basic and clinical research (Sayan et al., 2001). Apoptosis, or programmed cell death, is implicated in biological processes ranging from embryogenesis to aging. Apoptosis is one of
the most commonly used words in biology and medicine today with thousands of citations to date. It has been introduced into modern scientific writing by Kerr, Wyllie and Currie (1972) to describe a form of cell death distinct from necrosis (programmed cell death). Seemingly, apoptosis was chosen for its meaning of falling of leaves in ancient Greek (Kerr et al, 1972) and this has remained the accepted etymology in biomedical sciences. Apoptosis is a genetically regulated form of cell death and involves an orchestrated collapse of a cell, characterized by membrane blebbing, cell shrinkage, condensation of chromatin, and fragmentation of DNA, followed by rapid engulfment of the dead cell by neighboring macrophages. Apoptosis is distinguished from necrosis by the absence of an associated inflammatory response. The different types of cell death in the heart are compared in Table 1.

The function of apoptosis in regulating cardiovascular disease has been described as a possible mechanism explaining the pathophysiological significance of various forms of cardiovascular diseases (Rezvani et al., 2000; Russo & Russo, 2003).

TUNEL, or terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick end labeling, is a commonly used assay to detect apoptosis. This method labels the 3’-OH end of the nicked DNA fragments. The TdT method has been shown to enhance detection and analysis of apoptotic cells by flow cytometry (Timm et al., 1996). TUNEL has also been successfully used in co-localization
**Table 1**: Different types of cell death in the heart

<table>
<thead>
<tr>
<th>Features of cell death</th>
<th>Apoptosis</th>
<th>Necrosis</th>
<th>Autophagic</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Fragmentation</td>
<td>+</td>
<td>+</td>
<td>_</td>
</tr>
<tr>
<td>DNA Laddering</td>
<td>+</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>Mitochondrial damage</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Active caspase-3</td>
<td>+</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>Membrane blebbing</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Inflammation</td>
<td>_</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Chromatin condensation</td>
<td>+</td>
<td>_</td>
<td>_</td>
</tr>
</tbody>
</table>

*+ = Observed*

*- = Not observed*
studies with caspase-3 in E9 mouse embryos (Mirkes et al., 2001). Detection of cell death in cardiomyocytes by TUNEL assay alone is often questioned because of concerns regarding specificity and sensitivity (Ohno et al., 1998; Kumar & Jugdutt, 2003). Cardiomyocytes are mononuclear or binuclear, hence if only part of the fragmented nuclei or only one nucleus is stained with TUNEL assay it is difficult to determine if that cell is still functionally active (Ohno et al., 1998; Kang & Izumo, 2003; Kumar & Jugdutt, 2003).

The growing heart is susceptible to many genetic and environmental influences, but the myocardium seems relatively resistant to many insults (Poelmann et al., 2000; Mirkes et al., 2001). Apoptosis mainly occurs in the non-myocardial compartment of the embryonic heart (Poelmann et al., 2000), which consists of cells derived from the endocardium, the epicardium and the neural crest. Teratogen-induced cell death is selective; i.e., different cells and tissues within the developing embryo exhibit different sensitivities to the induction of cell death by teratogens (Mirkes et al., 2001). Cells of E9 rodent heart demonstrated resistance to the cell-death inducing potential of teratogens such as hyperthermia, cadmium, cyclophosphamide and deoxyadenosine (Mirkes et al., 2001). However, we have found that hypoglycemia induces cell death in E9.5 mouse heart (Ghatnekar & Smoak, 2001). Cardiomyocytes like other cells show similar characteristics of cell death.
Interactions between different pro- and anti-apoptotic molecules are complex. Apoptosis can be initiated by mitochondria, which releases cytochrome c into the cytosol in response to various extrinsic stimuli (Li et al., 1997). Glucose transport and metabolism serve as critical regulatory points in the early apoptotic cascade (Kan et al., 1994; Berridge et al., 1996 Johnson et al., 1996; Shim et al., 1998). Glucose deprivation or hypoglycemia leads to energy depletion and activates the mitochondrial death cascade (Moley & Mueckler, 2000).

Figure 2 demonstrates a typical cell death pathway in most mammalian cells. The mitochondrial pathway may be the most active pathway causing cell death in the embryonic heart in response to hypoglycemia.
Figure 2:
General overview of the apoptotic pathway in mammalian cells. The factors emphasized in this thesis are labeled in green.
**P53 in the embryonic heart**

The p53 gene encodes a 53 kDa nuclear phosphoprotein that is a negative regulator of the G1-S phase transition in the cell cycle. Tumor suppression is linked to a cell cycle checkpoint induced by DNA damage (Kastan et al., 1991), in which p53 can induce either growth arrest (Clark et al., 1993) or apoptosis (Lowe et al., 1993). p53 can function as a transcription factor, in which it activates the transcription of p21 (El-Diery et al., 1993). p21 is a protein that inhibits the activity of G1 cyclin-Cdk complexes (required for progression into the S-phase) as well as the activity of PCNA (required for DNA synthesis and repair). The critical role of p53 in the regulation of both growth and apoptosis is clearly demonstrated (Sherr, 2000; Vousden, 2000). In cardiomyocytes, forced expression of p53 results in apoptosis (Long et al., 1997; Kirshenbaum & De Moissac, 1997). This may be due to p53 up-regulating Bax protein which activates the mitochondrial cell death cascade (Fig 2).

Studies using p53 null mouse embryos illustrate the importance of this gene along with its mRNA and protein. Mdm2, an oncoprotein, can also maintain p53 expression at a low level by making it susceptible to proteolysis (Momand et al., 2000). Inactivation of p53 via homologous recombination in mice resulted in an increased incidence of neoplasia in many cell types (Donhower et al., 1992). Expression of p53 has not been consistently demonstrated in the developing mouse heart. No p53 expression was found in hearts of gd10 rat or gd8 mouse
embryos under normal or stressed conditions (Wubah et al., 1996; Dugyala et al., 2002). However, p53 expression was demonstrated in the hearts of chick, rat, and human embryos (Miosge et al., 1997; Lichnovsky et al., 1998; Rees et al. 1999; Krinka et al., 2001) and in response to hypoxia in chick cardiomyocytes (Chandel et al., 2000).

**Caspase-3 in the embryonic heart**

Caspase-3 is one of 14 cysteine proteases. Caspase-3 has been identified as a key mediator of apoptosis of mammalian cells (Kothakota et al., 1997). It is known to play a key role in the apoptotic pathway induced by a variety of stimuli (Faleiro et al., 1997). Effector caspases depend on initiator caspases for activation. The deletion of different caspases by homologous recombination has resulted in mice that exhibit various phenotypes, ranging from normal to embryonic lethality (Cardone et al., 1998; Zheng et al., 1999). Caspase-3 has been detected in the cytoplasm of all cells studied, including heart cells, where it exists as an inactive pro-enzyme of molecular weight 32 kDa (Mirkes et al., 2001). In response to various apoptotic stimuli, pro-caspase-3 is cleaved by other activated caspases, primarily caspase-8 (Tewari et al., 1995; Boldin et al., 1995; Fernandes-Alnemri, 1996) and caspase-9 (Li et al., 1997), to form two subunits (17 and 12 kDa), which then associate to form the active enzyme (heterotetramer). Once activated, caspase-3 cleaves a variety of substrates,
such as proteins involved in DNA replication, transcription and translation; cytoskeletal proteins; and kinases, phosphatases, and other caspases (Stroh & Schulze-Osthoff, 1998). In addition, active caspase-3 indirectly activates a nuclease (caspase activated nuclease) responsible for internucleosomal DNA fragmentation (Nagata, 2000).

Studies with teratogens such as hyperthermia, cyclophosphamide and sodium arsenite (Mirkes & Little, 1998) have demonstrated apoptosis by activation of caspase-3 and DNA fragmentation in early post-implantation mouse embryos with no evidence of apoptosis and active caspase-3 in the heart. However, we have demonstrated cell death is produced in the embryonic heart following exposure to hypoglycemia by TUNEL staining, Lysotracker® staining, and presence of active caspase-3 (Ghatnekar & Smoak, 2001; Ghatnekar et al., 2004).

Studies using specific inhibitors to block the activity of active caspase-3 are necessary to demonstrate the importance of caspase-3 in mediating cell death in the embryonic heart when exposed to hypoglycemia. Blocking or absence of, caspase-3 activity in the embryo has demonstrated dramatic reduction in cell death in the brain, but not in the limbs (Lee et al., 1999; Mirkes et al., 2001). Thus, studying the activity and importance of caspase-3 in mediating cell death in the embryonic heart will provide insights into the complex regulation of cell death. This understanding will eventually aid in understanding
the mechanisms of hypoglycemia-induced birth defects, and may help in the prevention of hypoglycemia-induced cardiac defects.

**Heat Shock Protein 70 (Hsp70) in the embryonic heart**

Hsp70 is part of a fairly large family of stress proteins, which are induced by cells in response to variety of external stimuli such as metabolic disturbances and injuries. Hsp proteins were first identified as a family of proteins produced in response to cellular stress such as heat shock, osmotic shock, nutrient deprivation, infectious agents, inflammation and malignancy (Ciupitu et al., 1998; Liu et al., 2000; Lee, 2001; Oglesbee et al., 2002).

Hsp proteins are highly conserved, abundantly expressed proteins with diverse functions (Welch, 1993). Hsp’s act as molecular chaperones and help maintain correct folding of newly synthesized polypeptides (Beckmann et al., 1990). In addition to important functions in protein refolding and transport, the Hsp70 family is also capable of binding and sequestering activated caspases, APAF and AIF making them good targets for a role in ischemia tolerance (Jaattela et al., 1998; Beere et al., 2000; Ravati et al., 2000; Saleh et al., 2000).

Overexpression of Hsc 70 (constitutive form) and Hsp70 (inducible form) protects cells from heat shock-induced cell death by preventing the processing of procaspases 9 and 3 (Mosser et al., 2000). Increased demand for the chaperoning function of Hsp70 occurs at different stages in a cells life, as seen
by changes in its level of expression during development and through the cell cycle. Stress-induced proteins like Hsp70 may also help determine the fate of cells faced with death (Landry et al., 1989; Li et al., 1991).

Several studies have demonstrated the effects of over-expression of Hsp70 in the embryonic heart (Mirkes et al., 1999; Abdelwahid et al., 2001). Hsp70 is the most abundantly induced Hsp in a variety of stress inducing scenarios. Hsp70 has shown to protect embryonic day 8 mouse embryos by inducing thermotolerance (Mirkes et al., 1999), and transfection of cells with Hsp70 gene demonstrated enhanced thermotolerance (Angelidis et al., 1991; Williams et al., 1993). Consistent with this function, microinjection of antibodies against Hsp70 into cells blocked the induction of thermotolerance (Riabowol et al., 1988). Hsp70 over-expressing transgenic mice have demonstrated that expression of Hsp70 increases the resistance of adult heart to ischemic injury (Marber et al., 1995; Plumier et al., 1995; Radford et al., 1996).

According to my preliminary studies Hsp70 is not up-regulated significantly in the E9.5 embryonic heart in response to 24 hr hypoglycemic exposure. Over-expression of Hsp70 in the embryonic heart exposed to hypoglycemia may prevent the occurrence of unnecessary cell death in the embryonic heart in response to hypoglycemia.
**Research overview**

The mechanisms by which hypoglycemia stops cell proliferation and induces cell death are currently unclear. Critical period for development of cardiac malformations occurs very early in pregnancy. It is therefore critical to understand the mechanisms by which these malformations may occur. This thesis helps uncover some a critical mechanism by which hypoglycemia induces congenital heart malformations.

The subsequent chapters present a detailed documentation of some of the challenges faced by the embryonic mouse heart due to brief exposure to hypoglycemia. The first chapter of this thesis demonstrates the use of NMR technique to study changes in glucose metabolism in the embryonic heart in response to hypoglycemia. Subsequent chapters are devoted to evaluation of the effects of hypoglycemia on induction of cell death at organogenesis in the embryonic mouse heart, which is resistant to other teratogenic insults. These chapters demonstrate the susceptibility of the embryonic heart to hypoglycemia-induced changes in cell proliferation and cell death and also look into possible mechanisms leading to cell death in the embryonic mouse heart. In addition, this thesis also examines possible mechanisms to help prevent or reduce the occurrence of unnecessary cell death in the embryonic heart as a result of hypoglycemic exposure.
CHAPTER 1

$^{13}$C-NMR study of hypoglycemia-induced glycolytic changes in embryonic mouse heart

Abstract

Glucose metabolites can be detected in embryonic mouse tissues using $^{13}$C-NMR spectroscopy. The advantage of this method is in its chemical specificity and the ability to follow metabolic changes. In this study, CD-1 mice were mated and embryos excised on gestational day (gd) 10.5 (plug = gd 0.5). Hearts were isolated and cultured in 150 mg/dl glucose (normoglycemic medium) or 40 mg/dl glucose (hypoglycemic medium) for 6 hours. $^{13}$C-labeled glucose comprised 62-64% of total glucose in the culture medium. Pre- and post-culture media were treated with $D_2$O, and $^{13}$C spectra were obtained using a Bruker Avance 500 MHz spectrometer operating at 11.744 tesla (125.7 MHz for $^{13}$C). NMR spectra demonstrated resonances for $^{13}$C-glucose in pre-culture normoglycemic and hypoglycemic media. Post-culture spectra for normoglycemic and hypoglycemic media demonstrated $^{13}$C-glucose signals, as well as a signal for $^{13}$C-lactate. Area under the curve (AUC) was measured for the [1-$^{13}$C-glucose] resonance from pre-culture media and the [3-$^{13}$C-lactate] resonance from post-
culture media. The ratios of AUC for post-culture [3-\textsuperscript{13}C-lactate] to pre-culture [1-\textsuperscript{13}C-glucose] were calculated and found to be higher in hypoglycemic than in normoglycemic media. Our results confirm earlier findings using radiolabeled substrates and suggest that \textsuperscript{13}C-NMR spectroscopy can be used to study glucose metabolism in isolated embryonic hearts exposed to hypoglycemia. NMR effectively measures glucose and its metabolite, lactate, in the same spectrum and thus determines metabolic flux in the isolated embryonic heart after exposure to hypoglycemia and normoglycemia. This method could evaluate glucose metabolism in embryonic tissues following other teratogenic exposures.
Introduction

An adequate supply of glucose to the embryonic heart during organogenesis is critical to normal development of cardiac function and metabolism. The embryo and embryonic heart rapidly use glucose during the period of organogenesis, and the vast majority of this substrate is metabolized to lactate by glycolysis for the production of ATP (Tanimura and Shepard, 1970; Clough et al., 1983; Akazawa et al., 1994). The embryonic heart appears to be particularly sensitive to alterations in glucose availability; changes in energy supply may contribute to cardiac dysmorphogenesis and abnormal cardiac function.

Hypoglycemia is a commonly noted condition in humans, particularly as a side effect of diabetes mellitus therapy (Nilsson et al., 1988), but also as a result of disease states such as starvation, tumors and others (Senior and Sadeghi-Nejad, 1989). Previous work in this laboratory has demonstrated that hypoglycemia affects the morphology, function and metabolism of the embryonic heart (Smoak, 1997). In addition, hypoglycemia increases the expression of the glucose transport protein, Glut-1 (Smoak and Branch, 2000), and a key glycolytic enzyme, hexokinase, in the embryonic mouse heart (Smoak et al., 1999). Previous studies in this laboratory have used radiolabeled substrate to measure
glucose metabolism in embryonic tissues (Smoak, 1997). Recent investigations have used biosensors (Palmisano et al., 2000), multienzyme sensors (Perdomo et al., 2000), and fiber optic sensors (Ignatov et al., 2001; Marquette et al., 2001) to detect glucose and its glycolytic metabolites in adult tissues in physiological and pathological conditions. In this study we demonstrate the effectiveness of nuclear magnetic resonance (NMR) spectroscopy to study glucose metabolism in the isolated embryonic heart exposed to hypoglycemia.

NMR spectroscopy is a method whereby the nuclei of certain atoms are immersed in a static magnetic field and exposed to a second oscillating magnetic field. NMR has been used to follow glycolytic kinetics and energy states in a variety of cells (Artimov et al., 1998; Sibson et al., 1998; Neves et al., 1999). The advantage of this method is in its chemical specificity and the ability to follow metabolic changes under physiological conditions. It is limited by the requirement for highly enriched precursors and sample label density to achieve adequate sensitivity. NMR spectroscopy has a wide range of applications and has been used to study effects of hypoxia and hypoglycemia on high-energy phosphate metabolites, such as ATP and phosphocreatine, in brain cells (Alves et al., 2000).

NMR is commonly used to study molecules that contain carbon. The carbon-12 nucleus, which comprises 98.9% of the carbon nuclei on earth, lacks a nuclear spin, whereas the carbon-13 \(^{13}\text{C}\) nucleus, which is 1.1% naturally available, has a nuclear spin, making \(^{13}\text{C}\)-NMR spectroscopy much less sensitive
than NMR spectroscopy for the highly abundant hydrogen. The problem of sensitivity may be overcome by using isotope enrichment and higher magnetic field strength. $^{13}$C-NMR may be used to detect levels of glucose and its metabolite, lactate, in the same spectrum. $^{13}$C-NMR has been used extensively to study glycolysis (Damico et al., 1996) and tricarboxylic acid (TCA) cycle kinetics (Carvalho et al., 2001) in the heart. This method thus allows determination of glycolytic flux in the embryonic heart in response to hypoglycemic and normoglycemic exposure.
Methods

Embryos:

Mouse embryos were obtained by mating randomly bred CD-1 mice overnight and checking for vaginal plugs the following morning, which was designated gestational day (gd) 0.5. Pregnant females were killed by cervical dislocation on gd 10.5 (30-somite stage) and the resulting embryos were dissected in Tyrode’s buffer under a stereozoom microscope. Hearts were isolated by severing outflow and inflow tracts and were then placed in culture medium.

Culture medium:

Culture medium consisted of 50% rat serum and 50% Tyrode’s buffer. Serum was obtained by immediate centrifugation of blood withdrawn from the abdominal aorta of ether-anesthetized male Sprague-Dawley rats. Hypoglycemic serum was obtained by injecting rats with 50 international units of insulin intraperitoneally two to three hours before blood withdrawal. Serum was heat inactivated, treated with antibiotics, and stored at −70°C until use. Powdered uniformly-labeled (U) $^{13}$C-glucose (Cambridge Isotope Laboratories Inc., MA, USA) was dissolved in 1.5 ml rat serum in a 10 ml sterile glass vial, and 1.5 ml Tyrode’s buffer was added to yield a final glucose concentration of 40-60 mg/dl.
(hypoglycemia) or 140-160 mg/dl (normoglycemia). Glucose concentration was monitored with a Beckman glucose analyzer, and $^{13}$C-glucose comprised 62 to 64% of the total glucose concentration.

Aliquots of 600 µl hypoglycemic and 600 µl normoglycemic pre-culture media were stored at 4°C. A volume of 100 µl deuterated water (D$_2$O) was added prior to NMR analysis.

**Culture of isolated hearts:**

Isolated gd 10.5 embryonic hearts were placed, 12 per vial, in 3 ml of $^{13}$C-enriched hypoglycemic or normoglycemic culture medium. Vials were aerated with a gas mixture containing 95% oxygen, then stoppered and placed on a wheel rotating at 30 rpm in a 37°C incubator. After a six hour exposure period, 600 µl aliquots of hypoglycemic and normoglycemic post-culture media were collected. A volume of 100 µl D$_2$O was added to each, and samples were stored on ice until NMR was performed.

**NMR analysis of samples:**

The $^{13}$C-NMR spectra were run on a 500 MHz Avance Bruker DRX spectrometer with an Oxford Narrow Bore Magnet, SGI INDY Host Workstation, XWINNMR Software version 2.5. The instrument was equipped with three frequency channels, a waveform memory and amplitude shaping unit, with a
three-channel gradient control unit (GRASP III), variable temperature unit, and a pre-cooling and temperature stabilization unit. A 5 mm ID probe (ID500-5EB, Nalorac Corp.) was used for preliminary measurements. The NMR probe was tuned to $^{13}$C frequency, which was 125.75 MHz in the 500 MHz spectrometer ($^1$H frequency = 500.128 MHz). All quantitative measurements were performed using a single frequency 5 mm carbon $^{13}$C-dedicated probe, which was Ge NMR adapted for Bruker room temperature shims.

**NMR sample preparation and instrumental parameters:**

Pre-culture and post-culture samples were dissolved twice in approximately 700 µl of H$_2$O/D$_2$O and transferred to a 5 mm NMR tube for analysis. Samples were run using standard NMR parameters (Table 2). All spectra were acquired at 298 Kelvin (25°C). Trimethyl silane was used as the internal standard.

The $^{13}$C spectra of pre-culture and post-culture media were obtained at a frequency of 125.75 MHz using the Nuclear Overhauser Effect (NOE) suppressed inverse-gated proton decoupling technique (Freeman et al., 1972; Martin et al., 1980; Kalinowski et al., 1988). A Waltz-16 composite pulse was used as the decoupling pulse. A relaxation reagent, Chromium (III) acetylacetonate [Cr(acac)$_3$] (0.03M), was added to the sample. This reduced $^{13}$C
$T_1$ relaxation to approximately 1.1 second and allowed a five second acquisition delay. All $^{13}$C spectra were collected using 20,000 scans.
Table 2. $^{13}$C-NMR parameters used in these experiments.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>$^{13}$C-NMR value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spectrometer frequency (MHz)</td>
<td>125.75</td>
</tr>
<tr>
<td>Spectral width (Hz) or (ppm)</td>
<td>30303 or 240</td>
</tr>
<tr>
<td>Number of data points</td>
<td>32,000</td>
</tr>
<tr>
<td>Relaxation Delay (s)</td>
<td>1.1</td>
</tr>
<tr>
<td>Acquisition time (s)</td>
<td>2.3</td>
</tr>
<tr>
<td>Pulse width ($\mu$s) and tip angle</td>
<td>14 at 90°</td>
</tr>
<tr>
<td>Number of transients</td>
<td>20,000</td>
</tr>
<tr>
<td>Number of dummy scans</td>
<td>16</td>
</tr>
</tbody>
</table>
Data analysis:

NMR spectra for pre- and post-culture media from five separate experiments were analyzed using NMR software. Area under the curve (AUC) was calculated for the [1-\textsuperscript{13}C-glucose] resonance in pre-culture media and for the [3-\textsuperscript{13}C-lactate] resonance in post-culture media. Ratio of AUC for [3-\textsuperscript{13}C-lactate] in post-culture media to [1-\textsuperscript{13}C-glucose] in pre-culture media was calculated for each experiment. Data were statistically analyzed with analysis of variance using SAS software at $p < 0.05$. This is a one way ANOVA model, which includes the main effects of treatment.
Results

The pre-culture NMR spectrum for normoglycemic medium (Fig. 3a) demonstrates resonances for the individual carbon atoms of $^{13}$C-glucose. A split resonance is seen for [1-$^{13}$C-glucose], which represents a doublet; one appears at 92.92 ppm and the second resonance at a distance of 45 Hz from the first (1ppm = 125.75 Hz). Doublets are due to carbon-carbon coupling in an enriched $^{13}$C-glucose sample. Carbon chemical shifts appear at 73.17 ppm for [2-$^{13}$C-glucose], 72.02 ppm for [3-$^{13}$C-glucose], 68.13 ppm for [4-$^{13}$C-glucose], 74.92 ppm for [5-$^{13}$C-glucose], and 60.92 ppm for [6-$^{13}$C-glucose]. A chemical shift for lactate carbon atoms is not seen in this spectrum (Fig. 3a).

The post-culture normoglycemic medium demonstrates the same resonances for $^{13}$C-glucose as seen in the pre-culture spectrum, in addition to a distinct NMR signal for [3-$^{13}$C-lactate] at 20.15 ppm (Fig. 3b).
Fig. 3. $^{13}$C-NMR spectra for $^{13}$C enriched normoglycemic medium.

a. Spectrum of pre-culture normoglycemic medium. Note resonances for $^{13}$C-labeled carbon atoms of glucose and the enlarged view of [1-$^{13}$C-glucose] doublet (insert).
b. Spectrum of post-culture normoglycemic medium. Note resonances for $^{13}$C-labeled carbon atoms of glucose and [3-$^{13}$C-lactate]. Inserts show enlarged views of AUC measurements for [1-$^{13}$C-glucose] and [3-$^{13}$C-lactate].
The pre-culture NMR spectrum for hypoglycemic medium (Fig. 4a) demonstrates the same chemical shifts for the individual carbons of $^{13}$C-glucose as those seen in pre-culture normoglycemic medium. A chemical shift for lactate carbon atoms is not seen in this spectrum (Fig. 4a).

The post-culture hypoglycemic medium demonstrates the same resonances for $^{13}$C-glucose as seen in the pre-culture spectrum. In addition, a distinct signal for [3-$^{13}$C-lactate] is present at 20.15 ppm (Fig. 4b) and has higher amplitude than the comparable signal in post-culture normoglycemic medium (Fig. 3b).

The average ratio of AUC values for post-culture [3-$^{13}$C-lactate] to pre-culture [1-$^{13}$C-glucose] from five separate experiments is two-fold higher in hypoglycemic medium compared to normoglycemic medium at $p<0.0035$ (Fig. 5).
Fig. 4. $^{13}$C-NMR spectra for $^{13}$C enriched hypoglycemic medium.

a. Spectrum of pre-culture hypoglycemic medium. Note resonances for $^{13}$C-labeled carbon atoms of glucose and the enlarged view of $[1-^{13}$C-glucose] doublet (insert).
b. Spectrum of post-culture hypoglycemic medium. Note resonances for $^{13}$C-labeled carbon atoms of glucose and [3-$^{13}$C-lactate]. Inserts show enlarged views of AUC measurements for [1-$^{13}$C-glucose] and [3-$^{13}$C-lactate].
Fig. 5.

Graph demonstrating average ratios + S.E.M. of post-culture [3-\(^{13}\)C-lactate] to pre-culture [1-\(^{13}\)C-glucose] AUC values from five separate experiments. Note two-fold higher AUC ratio in hypoglycemic medium vs. normoglycemic medium.

* Significantly different at \(p < 0.05\).
Discussion

In glycolysis, the 6-carbon sugar, glucose, produces two 3-carbon pyruvate molecules, which are converted to two 3-carbon molecules of lactate (Fig. 6). Lactate is the metabolic endpoint in glycolysis under anaerobic conditions in normal adult tissues but is the normal glycolytic end point of aerobic glucose metabolism in the organogenesis stage embryo. This pathway provides the necessary energy essential for embryonic growth and survival in early gestation. In this study, it is assumed that $^{13}$C-glucose is metabolized by the embryonic heart in a manner similar to that of $^{12}$C-glucose (Fig. 6), an assumption supported by NMR studies in other tissues.

The purpose of this study is to demonstrate that NMR spectroscopy may be effectively used to characterize glycolytic metabolism in the embryo and embryonic heart exposed to hypoglycemia in culture. In our preliminary NMR studies, gd 9.5 mouse whole embryos were found to produce higher lactate levels after exposure to hypoglycemic medium compared to those cultured in normoglycemic medium (unpublished data). Isolated hearts were used for the present study in order to assess the response of the isolated embryonic heart to hypoglycemia.
Fig. 6.

Schematic of glycolysis demonstrating disposition of $^{13}$C atoms from metabolism of U-$^{13}$C-glucose to $^{13}$C-lactate.

$\text{DHAP} = \text{dihydroxyacetone phosphate}; \text{G3P} = \text{glyceraldehyde-3-phosphate}$
Hypoglycemia has been shown to produce morphological, functional and metabolic changes in embryonic heart (Smoak, 1997) and to increase Glut-1 expression in embryonic heart (Smoak and Branch, 2000). Hypoglycemia also increases hexokinase activity (Smoak et al., 1999), which positively regulates the glycolytic pathway by converting more glucose to glucose-6-phosphate, resulting in increased lactate production. In this study, NMR spectra demonstrate a higher production of lactate in isolated embryonic hearts exposed to hypoglycemia as compared to normoglycemia. It is likely that increased transport of glucose through the cell membrane leads to increased glucose metabolism under hypoglycemic conditions.

The $^{13}$C-NMR method has been effectively used to study glucose metabolism in teratogen or drug induced neurological disorders (Cruz and Wolf, 2001). This method has been widely applied in the biological sciences to study phenomena ranging from chemical composition to molecular dynamics. In this study, we have measured $[3^{-13}$C-lactate] as an endpoint in the glycolytic pathway because lactate is not further metabolized, and the TCA cycle is not fully functional, at this stage of embryonic development. $[3^{-13}$C-lactate] released into the culture medium was used to estimate total lactate production by the embryonic hearts exposed to hypoglycemia and normoglycemia in vitro. The $[3^{-13}$C-lactate] in culture medium was analyzed because its concentration was higher than $[3^{-13}$C-lactate] in the embryonic hearts. This method could be used to
measure $^{13}$C-labeled glycolytic intermediates in isolated embryonic organs if they are pooled to provide adequate amounts of $^{13}$C-labeled intermediates.

The [1-$^{13}$C-glucose] resonances display doublets due to carbon-carbon splitting. The distance between these two [1-$^{13}$C-glucose] resonances is 45 Hz (1ppm = 125.75 Hz). The resonance for anomeric carbon atom 1 of glucose appears in the same region as those for an aromatic compound. The $^{13}$C resonances of glucose appear downfield from the [3-$^{13}$C-lactate] resonance. The [1-$^{13}$C-glucose] and [3-$^{13}$C-lactate] peaks were used for measurement in this study because these carbon atoms produced the most distinct resonances for AUC calculations and have been previously used successfully by other investigators (Cruz and Wolf, 2001).

Our results demonstrate that NMR spectroscopy, as can be performed in standard NMR facilities, effectively compares glucose metabolism in response to different glycemic conditions in the embryo and embryonic heart. The use of NMR spectroscopy for measuring glycolysis in embryonic tissues is better suited to in vitro than in vivo studies because of the substantial amount of $^{13}$C enriched glucose needed for maternal dosing. The present results use NMR methodology to confirm our previous findings that hypoglycemia increases glycolytic metabolism in the embryonic heart. We conclude that NMR spectroscopy may be effectively used to study glucose metabolism in isolated embryonic tissues exposed to teratogenic conditions such as hypoglycemia.
Abbreviations

gd = gestational day
AUC = area under the curve
Glut-1 = glucose transport protein 1
D₂O = deuterated water
NOE = nuclear overhauser effect
Hz = hertz
[1⁻¹³C-glucose] = carbon atom no. 1 of glucose
[3⁻¹³C-lactate] = carbon atom no. 3 of lactate
CHAPTER 2

Hypoglycemia Induced Changes in Cell Death and Cell Proliferation in Organogenesis Stage Embryonic Mouse Heart with Emphasis on E9.5

Abstract

Hypoglycemia is a side effect of diabetes therapy and causes abnormal heart development. While embryonic heart cells are largely resistant to teratogen-induced apoptosis, it is unknown what effects hypoglycemia has on cell survival in the developing heart. Hypoglycemia was tested for effects on cell death and cell proliferation in embryonic mouse heart cells by exposing mouse embryos on E8.5 – E11.5 to hypoglycemia in vivo or in vitro for 24 hr. Long-term effects of in vivo exposure on conceptus viability were evaluated at E18.5. Cell death was evaluated by: 1) two TUNEL assays in sectioned embryos to demonstrate DNA fragmentation; 2) confocal microscopy in whole embryos stained with Lysotracker®; 3) flow cytometry in dispersed E9.5 heart cells stained for TUNEL and MHC to quantify and characterize cell type susceptibility; and 4) immunohistochemistry (IHC) in sectioned embryos to evaluate potential involvement of caspase-3 active subunit and p53. Effects on cell proliferation were evaluated on E9.5 by IHC and Western analysis of proliferating cell nuclear
antigen (PCNA). My results show that in vivo hypoglycemic exposure on E9.5 reduced viability in conceptuses examined on E18.5. E8.5 hearts exposed to hypoglycemia for 24 hr demonstrated no significant increase in cell death, whereas E9.5 and E10.5 hearts demonstrated increased TUNEL and Lysotracker® staining. Flow cytometry in E9.5 heart cells demonstrated increased TUNEL-positive cells, and cells dual-labeled for TUNEL and MHC, in hearts of embryos exposed to hypoglycemia. Protein expression for p53 was increased in organogenesis stage embryonic heart. No change in caspase-3 active subunit was seen on E8.5, whereas a significant increase was seen on E9.5 and E10.5. PCNA was markedly reduced, in hearts of embryos exposed to hypoglycemia. These studies demonstrate that hypoglycemia reduces embryonic viability, induces significant cell death, and reduces cell proliferation in the E9.5 and E10.5 mouse heart, and these processes may involve active caspase-3 and p53.
Introduction

Congenital heart malformations contribute significantly to birth defects in the human population and are responsible for a large number of neonatal deaths. Little is understood regarding the pathogenesis of these defects. The embryonic heart demonstrates a dependence on glucose and glycolytic metabolism for the normal development of cardiac function and metabolism (Smoak, 1997). Glucose is necessary to maintain maximal heart rate under anaerobic conditions until embryonic day (E) 11 in rat embryos, followed by a shift to include extraglycolytic energy sources and aerobic metabolism at E13 (Cox and Gunberg, 1972a). The embryonic heart appears to be particularly sensitive to alterations in glucose availability, which may contribute to cardiac dysmorphogenesis and abnormal cardiac function.

Hypoglycemia is a common clinical condition in humans, particularly as a side effect of diabetes mellitus therapy (Nilsson et al., 1988) but also as a result of disease states such as starvation, tumors, and others (Senior and Sadeghi-Nejad, 1989). Hypoglycemia interferes with normal cardiac development and function because glucose is a major substrate for embryonic energy production and growth in early organogenesis (Cox and Gunberg, 1972b). In vivo and in vitro exposures to hypoglycemia have resulted in cardiac malformations in laboratory animals, even after brief periods (2 to 6 hr) of exposure (Buchanan et
In addition to morphological defects, alterations in glucose metabolism in the embryonic heart are produced by hypoglycemia; glycolysis is increased in mouse hearts exposed to 60 mg/dl glucose for ≥4 hr in vitro at E9.5 (Smoak, 1997).

Cells can die either by apoptosis or necrosis. Apoptosis or programmed cell death (PCD), occurs under physiological conditions and is an active, energy-consuming and irreversible process. Apoptosis is a genetically regulated form of cell death and involves the orchestrated collapse of a cell. Initial changes are in the nucleus, where the chromatin becomes condensed and DNA fragmentation occurs, while mitochondria and the surface membrane remain intact. This is followed by membrane blebbing to form apoptotic bodies and cell shrinkage, involving minimal macrophage or endothelial reaction (Kerr et al., 1972; Saraste and Pulkki, 2000). By contrast, necrosis is accidental cell death caused by gross cell injury, and it results in the death of groups of cells within a tissue. Necrosis involves an initial reversible cell swelling followed by irreversible cellular edema associated with mitochondrial thickening without initial alterations of the nucleus. After rupture of the cell membrane, the cell disintegrates and releases lysosomal enzymes, often evoking a strong inflammatory reaction (Majno and Joris, 1995; Zimmermann and Green, 2001).

Studies with several teratogens, such as hyperthermia, cyclophosphamide, sodium arsenite, cadmium, and deoxyadenosine, have demonstrated apoptosis
due to the activation of caspase-3 and DNA fragmentation in early post-implantation mouse embryos that have no evidence of cell death, apoptosis, or active caspase-3 in the heart (Mirkes and Little, 1998; Umpierre et al., 2001). Deoxycoformycin-induced accumulation of 2'-deoxyadenosine produced cell death in day 7–8 mouse embryos in most embryonic tissues except the heart and extra-embryonic membranes (Gao et al., 1994). The developing heart is susceptible to apoptosis caused by certain genetic and environmental influences, but the myocardium is relatively resistant to these insults. Apoptosis occurs mainly in the non-myocardial compartment of the embryonic heart (Poelmann et al., 2000), which consists of cells derived from the endocardium, epicardium, and neural crest. However, there is increasing evidence that cardiomyocyte apoptosis occurs in various disease conditions (Saraste and Pulkki, 2000). It has been demonstrated that neonatal cardiomyocytes undergo apoptosis through the caspase pathway under glucose deprivation (Bialik et al., 1999). The low incidence of cardiomyocyte apoptosis makes quantification difficult and requires sensitive methods of detection (Grosse and Manns, 1993; Veinot et al., 1997; Saraste et al., 1999).

Caspase-3 is one of 14 cysteine proteases and has been identified as a key mediator of apoptosis in mammalian cells (Kothakota et al., 1997). It is known to play an important role in the apoptotic pathway induced by a variety of stimuli (Faleiro et al., 1997). Caspase-3 has been detected in the cytoplasm of all
cells studied, including heart cells, where it exists as an inactive pro-enzyme of molecular weight 32 kDa. In response to various apoptotic stimuli, pro-caspase-3 is cleaved by other activated caspases, primarily caspase-8 (Boldin et al., 1995; Tewari et al., 1995; Fernandes-Alnemri et al., 1996) and caspase-9 (Li et al., 1997), to form two subunits (17 and 12 kDa), which then associate to form the active enzyme (heterotetramer). Once activated, caspase-3 cleaves a variety of substrates, such as: 1) proteins involved in DNA replication, transcription, and translation; 2) cytoskeletal proteins; and 3) kinases, phosphatases, and other caspases (Stroh and Schulze-Osthoff, 1998). Caspase-3 plays an important role in several key events during apoptosis, such as nuclear fragmentation, DNA fragmentation, and membrane blebbing, in a cell type- and stimulus-specific manner (Porter and Janicke, 1999; Nagata, 2000).

Cardiac morphogenesis is regulated by coordination of cell transformation, cell proliferation, and cell death. Cell proliferation is a tightly regulated process that is intimately associated with growth, differentiation, and tissue turnover. Proliferating cell nuclear antigen (PCNA) is a 35 kDa acidic nuclear protein that is expressed only in proliferating cells and thus can be used as a selective marker of proliferation in cell populations and tissues (Mathews et al., 1984). A PCNA antibody can be used to demonstrate levels of cellular proliferation by Western analysis or tissue distribution of cell proliferation by immunohistochemistry (IHC). Studies using IHC for PCNA demonstrated cell proliferation throughout the chick
embryonic heart, including the myocardium, on days 4–8 (Keyes and Sanders, 1999).

The p53 gene encodes a 53 kDa nuclear phosphoprotein that is a negative regulator of the G1-S phase transition in the cell cycle. Tumor suppression is linked to a cell cycle checkpoint induced by DNA damage (Kastan et al., 1991), in which p53 can induce either growth arrest (Clarke et al., 1993) or apoptosis (Lowe et al., 1993). Inactivation of p53 via homologous recombination in mice results in an increased incidence of neoplasia in many cell types (Donehower et al., 1992). p53 can function as a transcription factor by activating the transcription of p21 (El-Diery et al., 1993). p21 inhibits the activity of the G1 cyclin-Cdk complexes, which are required for progression into the S-phase, as well as PCNA, which is involved in DNA synthesis and repair. The critical role of p53 in the regulation of both proliferation and apoptosis is clearly demonstrated in the embryo (Wubah et al., 1996; Sherr, 2000; Vousden, 2000). Expression of p53 has not been consistently demonstrated in the developing heart. No p53 expression was found in hearts of gd10 rat or gd8 mouse embryos under normal or stressed conditions (Wubah et al., 1996; Dugyala et al., 2002). However, p53 expression was demonstrated in the hearts of chick, rat, and human embryos (Miosge et al., 1997; Lichnovsky et al., 1998; Rees et al. 1999; Krinka et al., 2001) and in response to hypoxia in chick cardiomyocytes (Chandel et al., 2000).
In cultured neonatal cardiomyocytes, forced expression of p53 results in apoptosis (Kirshenbaum and De Moissac, 1997; Long et al., 1997). It is therefore proposed that p53 is involved in teratogenic exposures that produce decreased proliferation and/or increased cell death in the embryonic heart. This study was undertaken to characterize the immediate and long-term effects of hypoglycemia on the embryo and embryonic heart. The induction of cell death by hypoglycemia in the embryonic heart was evaluated using two TUNEL methods and Lysotracker staining, and cardiomyocyte cell death was evaluated by flow cytometry. The effect of hypoglycemia on cell proliferation, as indicated by PCNA, was evaluated by IHC and Western analysis. Active caspase-3, total p53, and phosphorylated p53 were evaluated by IHC as potential mediators of hypoglycemia-induced changes in apoptosis and cell proliferation in the embryonic heart.

This investigation is a comparative study of susceptibility to hypoglycemia-induced apoptosis in the embryonic mouse heart during E8.5 to E11.5. This study will help us understand the effects of 24 hr hypoglycemia on the embryonic heart at E8.5, the 10 somite stage, E9.5, the 20 somite stage, and at E10.5, the 30 somite stage. The embryonic heart starts looping around the 10 somite stage, and undergoes septation and valvulogenesis around the 30-somite stage, hence any insult during this period of development is likely to cause some septal defects in the embryonic heart.
A better understanding of the effects of hypoglycemia on E8.5 to E11.5 will help us understand its effects on early embryonic heart development.
Materials and Methods

Mouse Embryos

Outbred CD-1 mice were maintained in a 12 hr light/dark cycle and provided food and water ad libitum. Mouse embryos were obtained by mating randomly bred CD-1 mice overnight and checking for vaginal plugs the following morning, which was designated E0.5. Embryos used for morphological assessments were obtained by mating CD-1 male mice, containing a TIE-2-LacZ transgene, with normal CD-1 females.

Hypoglycemic Exposure

In vitro. The whole embryo culture (WEC) method was used to expose mouse embryos to hypoglycemic and control medium in vitro. Pregnant females were killed by cervical dislocation on E8.5 (10-somite stage), E9.5 (20-somite stage) or E10.5 (30-somite stage), and embryos were prepared for WEC using an adaptation of methods originally described by New (1978). Embryos with an intact visceral yolk sac and ectoplacental cone were placed in culture medium composed of 75% rat serum and 25% Tyrode’s buffer. Serum was obtained by immediate centrifugation of blood withdrawn from the abdominal aorta of ether-anesthetized male Sprague-Dawley rats. Hypoglycemic serum was obtained by injecting rats with 50 international units (IU) insulin (Regular Iletin II Pork insulin;
Eli Lilly, Indianapolis, IN) intraperitoneally 2–3 hr before blood withdrawal. Serum was heat inactivated, treated with antibiotics, and stored at −20°C until use. Hypoglycemic medium (40 mg/dl glucose) was prepared with glucose-free Tyrode’s buffer, and normoglycemic medium (150 mg/dl glucose) was prepared by adding concentrated (50 mg/ml) _D-glucose. Two embryos were placed in 4 ml medium in 30 ml vials. The vials were aerated at the start of culture and at 12 hr with a gas mixture containing 5% oxygen for E8.5 embryos, 20% oxygen for E9.5 embryos, and 95% oxygen for E10.5 embryos, stoppered, and placed on a wheel rotating at 30 rpm in a 37°C incubator. Glucose was added at 12 hr to maintain appropriate levels in hypoglycemic and control media.

**In vivo.** CD-1 pregnant mice were injected subcutaneously (SC) on E8.5, E9.5 or E10.5 with 35 IU/kg ultralente insulin (Humulin U human insulin; Eli Lilly) in 100 µl saline. Controls were injected with 100 µl saline. Serum glucose levels for each mouse were monitored pre-injection and at 2, 4, 6, 12, and 24 hr post-injection with a Beckman Glucose Analyzer II (Beckman Instruments, Inc., Galway, Ireland). Treated mice were allowed access to food for 30 min after each injection, and water was provided throughout the treatment period. Control mice were allowed food and water ad libitum. Insulin was subsequently administered at 15-20 IU/kg SC to the treated mice, according to their blood glucose levels, in order to maintain 30-50 mg/dl serum glucose levels throughout the treatment period.
Morphological Assessments

Embryos were removed from WEC (in vitro exposure) or from pregnant dams (in vivo exposure) after 24 hr and examined for morphological features, including overall appearance and cardiac structure. Transgenic embryos carrying a TIE-2-LacZ transgene were fixed in a LacZ fixative solution (37% formaldehyde, 50% glutaraldehyde, 10% NP40, in 1X phosphate buffered saline [PBS]) for 30 min at 4°C, and exposed to 2.5% X-gal solution overnight at 37°C to stain endothelium. Hearts from CD-1 mouse embryos were pooled and sonicated using 10 µl per heart of sonication buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA) with protease inhibitor cocktail (Sigma, St. Louis, MO) added 1:100, then centrifuged for 1 min at 12,000 rpm at room temperature (RT), and the supernatant stored at -20°C. A Bradford (1976) assay, using Bio-Rad protein assay dye reagent concentrate (Bio-Rad Laboratories, Hercules, CA) and bovine serum albumin (BSA) standards, was used to determine total cardiac protein of whole heart homogenates from embryos exposed in vitro to hypoglycemia compared to controls. Total cardiac protein was calculated as µg per heart.

Eight (four treated on E9.5, and four control) pregnant mice were maintained on normal food and water until E18.5 when they were sacrificed by cervical dislocation, and fetuses were removed and examined for viability.

DNA Fragmentation Assays
DNA fragmentation is a commonly used marker for cell death. Embryos were fixed in 4% paraformaldehyde for 24 hr, paraffin embedded and sectioned sagittally at 5 μm. DNA fragmentation was evaluated using terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick end labeling (TUNEL). A fluorescent TUNEL assay was performed using the standard protocol (Roche Diagnostics, Indianapolis, IN), in which the TdT enzyme labels nicks in DNA at the 3’-OH end. The sections were visualized using a fluorescent microscope. The colorimetric CardioTACS assay was performed on E9.5 embryonic heart sections using the standard protocol (R&D Systems, Minneapolis, MN), in which a blue stain labels nicks in DNA at the 3’-OH end. CardioTACS reagents are supplemented with a cation that specifically enhances the labeling of cardiac cells. The sections were visualized using a light microscope. Positive and negative controls were included for both TUNEL assays, as described in each protocol.

**Lysotracker Staining**

Lysotracker is a vital stain for lysosomes that, because of their acidity, become more apparent after engulfing apoptotic bodies. Embryos were stained according to previously described methods (Zucker et al., 1999). Briefly, embryos were removed from WEC (in vitro exposure) or from pregnant dams (in vivo exposure), dissected free of surrounding membranes, and placed in 5 mM
Lysotracker in Hank’s balanced salt solution (HBSS) for 30 min at 37°C. Embryos were rinsed in cold 1X PBS and fixed in 4% paraformaldehyde for 24 hr in the dark at 4°C. Embryos were dehydrated with methanol and cleared with 1:1 benzyl alcohol and benzyl benzoate (BABB) for 1 hr. Embryos were placed in BABB in depression slides, coverslipped, and imaged using a Nikon C-1 (Nikon Instruments Inc., Melville, NY) confocal microscope.

**Flow Cytometry**

Flow cytometry was used to quantify and characterize apoptotic cell types in hearts of E9.5 embryos exposed in vivo to hypoglycemia compared to controls. Embryonic hearts were isolated by severing inflow and outflow tracts, and cardiac cells were dissociated in 0.05% trypsin at 37°C for 10 min. Trypsin was then inactivated using twice the volume of 10% fetal calf serum. Dissociated cells and residual tissue were resuspended in 2.5 ml HBSS and further dissociated in 300 IU/ml collagenase in HBSS for 15 min at 37°C. Cells were fixed with 1% paraformaldehyde, permeabilized with saponin buffer (0.1% v/v in 1X PBS) for 10 min at RT, and stained for DNA fragmentation using TUNEL Cell Death Detection kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. Dual staining for cardiomyocytes was performed using myosin heavy chain (MHC) goat polyclonal primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:50 in 1X PBS for 1 hr at RT. Cells were
washed and incubated with Texas red conjugated bovine anti-goat secondary antibody (Santa Cruz Biotechnology) at 1:500 in 1X PBS for 1 hr at RT. Flow cytometry was performed using a Becton Dickinson FACS Calibur (Becton Dickinson, San Diego, CA), and data obtained from four separate experiments were analyzed using Cell Quest software.

**Immunohistochemistry**

Embryos removed from WEC (in vitro exposure) or from pregnant dams (in vivo exposure) were fixed in 4% paraformaldehyde for 24–48 hr, paraffin embedded, sectioned sagittally at 5 µm, and placed on slides. Sections were deparaffinized in xylene, rehydrated in decreasing ethanol and 1X PBS, and permeabilized with saponin buffer (0.1% v/v in 1X PBS) for 10 min at RT. Antigen retrieval was performed with 2 N HCL at 37°C for 15 min. IHC analysis was performed for caspase-3 active subunit, which is commonly associated with increased apoptosis, and p53 and PCNA, two proteins associated with decreased cell proliferation. Negative controls for each protein were treated with 1X PBS instead of primary antibody.

**Active caspase-3.** Sections were stained for caspase-3 active subunit by incubating with rabbit polyclonal anti-active caspase-3 antibody (Promega, Madison, WI) at a 1:250 dilution in PBS with 2% BSA overnight at 4°C. Sections
were washed in PBS and incubated with biotinylated anti-rabbit IgG Link (BioGenex, San Ramon, CA) for 30 min at RT or fluorescently labeled donkey anti-rabbit secondary antibody. Sections were washed in PBS and incubated with peroxidase conjugated streptavidin (BioGenex) for 30 min. 3-amino-9-ethylcarbazole (AEC) in N,N-dimethylformide (Biogenex Laboratories, San Ramon, CA) chromogen was used as a substrate for the colorimetric reaction. Sections were counterstained with hematoxylin, coverslipped using GVA (Zymed, San Francisco, CA) mounting medium, and imaged with a light microscope.

**p53.** Sections were stained for total p53 using mouse monoclonal or rabbit polyclonal anti-p53 antibodies (DO-1, FL-393; Santa Cruz Biotechnology), each at 1:100 dilution in PBS, for 1 hr at 37°C. Sections were also stained for phosphorylated p53 using a rabbit polyclonal anti-p-p53 antibody (Ser 15-R; Santa Cruz Biotechnology), which labels a peptide containing phosphorylated Ser-15, at 1:200 dilution in PBS for 1 hr at 37°C. Sections were incubated with biotinylated link (BioGenex) for 45 min at RT. Sections were further processed and imaged as described above.

**PCNA.** Sections were stained for PCNA using Zymed kit (Zymed Laboratories, South San Francisco, CA) and standard staining protocol.

*Western Analysis*
Isolated hearts were pooled (approximately 15 to 20 hearts were exposed to hypoglycemia per experiment and 12 to 15 hearts were exposed to normoglycemia per experiment), sonicated in sonication buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA), and analyzed for total protein concentration using the Bradford (1976) method and BSA standards. Proteins (10 µg per sample) were resolved on a 4–15% gradient gel at 200 volts and transferred to a polyvinylidene fluoride (PVDF) (Bio-Rad Laboratories, Hercules, CA) membrane at 100 volts for 1 hr. The membrane was blocked with 5% non-fat dry milk in TTBS for 1 hr, and probed with primary antibody. The membrane was washed in TTBS and exposed to peroxidase labeled secondary antibody (Amersham International Plc, Amersham, UK) for 1 hr. The membrane was washed and exposed to chemiluminescence substrate ECL (Amersham Pharmacia Biotech UK, Buckinghamshire, UK). Membranes were stripped and re-probed for actin as a loading control. Membranes were imaged using a UVP Epi Chemi II darkroom (UVP Products, Upland, CA) or wrapped in plastic and exposed using x-ray film. Bands were scanned and densities quantified as a ratio of target protein to actin.

**Active caspase-3.** Membranes were probed using 1:1000 concentration of anti-active caspase-3 rabbit polyclonal primary antibody (Promega) overnight at 4°C and 1:100,000 goat anti-rabbit secondary antibody for 1 hr at RT.
p53 and p-p53. Membranes were probed with a 1:100 concentration of anti-p53 or anti-p-p53 rabbit polyclonal primary antibody, as for IHC, for 1 hr at RT and 1:10,000 goat anti-rabbit secondary antibody.

PCNA. Membranes were probed with a 1:200 concentration of anti-PCNA rabbit polyclonal primary antibody (FL-261; Santa Cruz Biotechnology) for 1 hr at RT, followed by 1:10,000 goat anti-rabbit secondary antibody. Membranes were wrapped in plastic and exposed using x-ray film. Bands were scanned and densities quantified as volume percent using densitometry.
**Data Analysis**

The data were collected by manually counting the number of TUNEL and Lysotracker stained cells from sections of embryos exposed to hypoglycemia (n = 3) or normoglycemia (n = 3). Cell count data was analyzed using Student’s t-test (Statistical research methods in the life sciences by P.V. Rao).

Numerical data from Western analysis were analyzed using analysis of variance. This is a one way ANOVA model, which includes the main effects of treatment. Tukey’s test was used to determine between-group differences using SAS software (SAS Inc., Cary, NC). A significance level of $p \leq 0.05$ was maintained throughout.
Results

*Morphological Effects of Hypoglycemia*

Embryos exposed to hypoglycemia or normoglycemia (controls) in vitro or in vivo were evaluated for morphological differences. Control embryos placed in WEC at E8.5, E9.5 or E10.5 and cultured for 24 hr in vitro, or allowed to develop in vivo during the same period, expanded in size and vascularity by E10.5. Overall apparent embryonic size, including the heart, was smaller in embryos exposed to hypoglycemia compared to controls (Fig. 7, 8, 9). The number of somites are same in treated and control embryos, indicating that at least some developmental processes are proceeding in embryos exposed to hypoglycemia. However, because the treated embryos are smaller in appearance some developmental processes must be perturbed by hypoglycemia. In general, embryos exposed to hypoglycemia were viable, but typically had a slow heart beat, pericardial edema, poor blood circulation in yolk sac vessels, and increased microvascular branching in embryonic blood vessels, compared to controls. These effects on size, viability, pericardial edema, and vascularity were consistent between embryos exposed to hypoglycemia in vivo and in vitro. Total cardiac protein was significantly lower in embryos exposed to hypoglycemia for 24 hr in vitro compared to controls (Fig. 10).
Figure 7. Morphological effects of hypoglycemia on E8.5 (10-somite stage) embryos.

E9.5 mouse embryos with TIE-2-LacZ transgene demonstrating reduced overall size of embryo after exposure on E8.5 for 24 hr to hypoglycemia (A) compared to control (B). H = heart.
Figure 8. Morphological effects of hypoglycemia on E9.5 (20-somite stage) embryos.

E10.5 mouse embryos with TIE-2-LacZ transgene demonstrating reduced size of embryo and heart (h) after exposure on E9.5 for 24 hr hypoglycemia (A) compared to control (B) medium.
Figure 9. Morphological effects of hypoglycemia on E10.5 (30-somite stage) embryos.

E11.5 mouse embryos with TIE-2-LacZ transgene demonstrating reduced overall size of embryo after exposure on E10.5 for 24 hr to hypoglycemia (A) compared to control (B).
Figure 10.

Bar graph demonstrating significant decrease in mean total cardiac protein in hearts of E9.5 embryos exposed to hypoglycemia (n = 5) (hypo) compared to controls (n = 5) (normo), measured by Bradford assay. *p ≤ 0.05, bars = SEM.
During in vivo exposures, maternal glucose levels were maintained for 24 hr at 30–50 mg/dl glucose in insulin treated mice, compared to 120–150 mg/dl glucose in controls (Fig. 11).

Fifty percent (two of four) of the pregnant mice evaluated on E18.5, after 24-hr hypoglycemic exposure on E9.5, demonstrated resorption of all embryos with an average resorption rate of 84.5% in treated mice, compared to no resorptions in the controls (Table 3, Fig. 12). Viable fetuses were smaller than controls, but otherwise appeared grossly normal. The size and appearance of resorption sites suggested that embryonic death occurred shortly after the 24-hr hypoglycemic exposure period.

Table 3.
Resorption Data from E18.5 Pregnant Dams Exposed to Hypoglycemia or Control Conditions on E9.5 for 24 hr

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Resorptions (%) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypoglycemia</td>
<td>4</td>
<td>84.5 ± 10.0 *</td>
</tr>
<tr>
<td>Control</td>
<td>4</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

n = no. of pregnant dams, *p ≤ 0.05.
Figure 11.

Graph depicting mean serum glucose levels during a 24 hr treatment period, demonstrating 30-50 mg/dl glucose in treated (dashed line) mice and 120-150 mg/dl glucose in controls (solid line). N = 7 experiments, bars = SEM.
Figure 12.

A. Uterus from mouse exposed to 24 hr hypoglycemia in vivo on E9.5 and sacrificed on E 18.5 showing complete resorptions of embryos, B. Uterus of mouse exposed to control conditions in vivo on E9.5 and sacrificed on E18.5 showing normal uterus with normally developing fetuses.
**DNA Fragmentation**

**E8.5 x 24 hr hypoglycemia.**

The fluorescent TUNEL assay (Fig. 13 A, B, C) did not reveal a significant difference in cell death in hearts of E8.5 mouse embryos exposed to 24 hr hypoglycemia in vivo or in vitro compared to controls.

**E9.5 x 24 hr hypoglycemia.**

The fluorescent TUNEL assay demonstrated increased intensity and number of fluorescently-labeled cells in hearts of embryos exposed to hypoglycemia compared to controls. TUNEL-positive cells were observed in most areas of the embryonic heart, including the myocardial layer and mesenchyme. Intense TUNEL staining was also noted in the proepicardial organ in embryos exposed to hypoglycemia (Fig. 14 A, B, G).

The colorimetric CardioTACS assay demonstrated an increased number of blue stained cells in heart sections of embryos exposed to hypoglycemia compared to controls (Fig. 14 C, D), with a staining pattern in the heart similar to that seen with fluorescent TUNEL. Positive and negative controls for both TUNEL assays demonstrated intense and absent staining, respectively (not shown). The results of fluorescent and colorimetric TUNEL assays were consistent between embryos exposed in vivo and in vitro to 24-hr hypoglycemia.
E10.5 x 24 hr hypoglycemia.

The fluorescent TUNEL assay (Fig. 15 A, B, C) demonstrated increased intensity and number of fluorescently-labeled cells in hearts of E10.5 mouse embryos exposed to 24 hr hypoglycemia in vivo and in vitro compared to controls.

Lysotracker®

E8.5 x 24 hr hypoglycemia.

The fluorescent Lysotracker® assay (Fig. 13 D, E, F) did not reveal a significant difference in cell death in hearts of E8.5 mouse embryos exposed to 24 hr hypoglycemia in vivo or in vitro compared to controls.

E9.5 x 24 hr hypoglycemia.

The Lysotracker assay revealed increased staining for cell death in cardiomyocyte and mesenchymal layers of hearts of whole embryos exposed to hypoglycemia compared to controls (Fig. 14 E, F, H). Staining was also present in the proepicardial organ in embryos exposed to hypoglycemia. These findings were consistent between embryos exposed in vivo and in vitro to 24-hr hypoglycemia.

E10.5 x 24 hr hypoglycemia.

The fluorescent Lysotracker® assay (Fig. 15 D, E, F) demonstrated increased intensity and number of fluorescently-labeled cells in hearts of E10.5 mouse
embryos exposed to 24 hr hypoglycemia in vivo and in vitro compared to controls.
Figure 13 (A, B, C).

No difference in TUNEL staining is observed between E8.5 embryonic hearts exposed to hypoglycemia (n = 3) (A) compared to control (n = 3) (B). C.
Quantitation of TUNEL-positive cells in total cardiac cell population in hypoglycemic & normoglycemic hearts, demonstrates no statistically significant difference in number of TUNEL positive stained cells between hypoglycemia and control. Magnification = 100x, h = heart, bars = SDM, Hypo = treated, Normo = control.
Figure 13 (D, E, F).

No difference in Lysotracker® red staining is observed between E8.5 embryonic hearts exposed to hypoglycemia (n = 3) (D) compared to control (n =
3) (E). F. Quantitation of Lysotracker®-positive cells in total cardiac cell population in hypoglycemic & normoglycemic hearts, demonstrates no statistically significant difference in number of Lysotracker® positive stained cells between hypoglycemia and control. Magnification = 100x, v = ventricle bars = SDM, Hypo = treated, Normo = control.
**Figure 14 (A-F).**

**A–D:** Fluorescent and colorimetric TUNEL assays demonstrating DNA fragmentation in 5 μm paraffin-embedded sections of embryos exposed on E9.5 to 24-hr hypoglycemia compared to controls. Number of green fluorescently labeled cells in the heart was increased in treated embryos (A) compared to
controls (B). Number of CardioTACS blue stained cells in the heart was increased in treated embryos (C) compared to controls (D).

**E, F**: Lysotracker staining of whole embryos visualized using a confocal microscope. Lysotracker staining was intense in the heart of treated embryos (E) compared to controls (F). Magnification = 100x, (pe) = proepicardial organ, (v) = ventricle.
Figure 14 G.

G: Quantitation of apoptotic cells in hearts analyzed by TUNEL assay. A significant increase in cell death is demonstrated in total cardiac cell population in E9.5 hearts exposed to hypoglycemia (n = 3), as compared to controls (n = 3). *p ≤ 0.05, bars = SDM.
**Figure 14 H.**

**H**: Quantitation of apoptotic cells analyzed by Lysotracker® assay. A significant increase in cell death is demonstrated in total cardiac cell population in E9.5 hearts exposed to hypoglycemia (n = 3), as compared to control (n = 3). *p ≤ 0.05, bars = SDM.
Figure 15 (A, B, C).

A dramatic increase in TUNEL staining is observed between E10.5 embryonic hearts exposed to hypoglycemia (n = 3) (A) compared to control (n = 3) (B). C. Quantitation of TUNEL-positive cells amongst total cardiac cell population in hypoglycemic & normoglycemic hearts, demonstrates statistically
significant difference in number of TUNEL positive stained cells between hypoglycemia and control. Magnification = 100x, a = atria, v = ventricle \( p \leq 0.05 \), bars = SDM, Hypo = treated, Normo = control.
Figure 15 (D, E, F).
A dramatic increase in Lysotracker® staining is observed between E10.5 embryonic hearts exposed to hypoglycemia (n = 3) (D) compared to control (n = 3) (E). F. Quantitation of Lysotracker®-positive cells amongst total cardiac cell population in hypoglycemic & normoglycemic hearts, demonstrates statistically significant difference in number of Lysotracker® red positive stained cells between hypoglycemia and control. Magnification = 100x, v = ventricle, *p ≤ 0.05, bars = SDM, Hypo = treated, Normo = control.
Flow Cytometry

Flow cytometry demonstrated an increased percentage of TUNEL-positive total cardiac cells from embryos exposed in vivo to hypoglycemia compared to controls (Fig. 16 A, B). Dual staining with TUNEL and myosin heavy chain (MHC) demonstrated a significantly higher percentage of TUNEL-positive/MHC-positive cells from embryos exposed to hypoglycemia compared to controls (Fig. 16 C, D). No significant difference was observed between the percentage of TUNEL-positive/MHC-positive cells and TUNEL-positive/MHC-negative cells in response to hypoglycemia (data not shown).
Figure 16.
Results of flow cytometry on cardiac cells from embryos exposed in vivo on E9.5 to 24-hr hypoglycemia compared to controls. A: Histogram demonstrating increased TUNEL-positive cardiac cells from treated embryos (dark line) compared to control (shaded region). A shift to the right of the dark line indicates increased percentage of cells stained for TUNEL. B: Graphical representation of mean data from A. \( n = 4, \) *\( p \leq 0.05, \) bars = SEM. C: Histogram of TUNEL and MHC dual labeling, demonstrating increased percentage of dual stained cardiac cells from treated embryos (dark line) compared to controls.
(shaded region). **D:** Graphical representation of data from C. $n = 4$, *$p \leq 0.05$,
bars = SEM, hypo = treated, normo = control.
Active Caspase-3

E8.5 x 24 hr hypoglycemia.

Immunohistochemistry was used to assess active caspase-3 levels in response to hypoglycemia, and demonstrated no change in active caspase-3 subunit expression in hearts of E8.5 mouse embryos exposed to 24 hr hypoglycemia in vivo or in vitro compared to controls (Fig. 17).

E9.5 x 24 hr hypoglycemia.

Immunohistochemistry demonstrated increased active caspase-3 subunit expression in hearts of embryos exposed to hypoglycemia compared to controls (Fig. 18 A, B). Cardiac cells of control embryos demonstrated minimal staining for active caspase-3, whereas staining was prominent in embryos exposed to hypoglycemia and was observed in the myocardial and mesenchymal layers of the embryonic heart. Negative controls demonstrated no staining (not shown).

Western analysis results were similar to those obtained by IHC and demonstrated a significant increase in active caspase-3 in hearts of mouse embryos exposed to 24-hr hypoglycemia compared to controls (Fig. 18 G, H).

E10.5 x 24 hr hypoglycemia.

Immunohistochemistry demonstrated a significantly increased amount of active caspase-3 subunit expression in hearts of E10.5 embryos exposed to 24 hr hypoglycemia compared to controls (Fig. 19). Negative controls demonstrated no staining (not shown).
p53

E8.5 x 24 hr hypoglycemia.

Immunohistochemistry was also used to assess whether changes in p53 expression occurs in response to hypoglycemia. These data demonstrated a prominent increase in total p53 protein expression in hearts of E8.5 embryos exposed to 24-hr hypoglycemia compared to controls (Fig. 20). Staining for total p53 was mostly nuclear, with a few cells showing cytosolic staining, and was observed in the myocardial and mesenchymal layers of the embryonic heart of embryos exposed to 24-hr hypoglycemia.

E9.5 x 24 hr hypoglycemia.

Immunohistochemistry demonstrated a prominent increase in total p53 protein expression in hearts of embryos exposed to 24-hr hypoglycemia compared to controls (Fig. 18 C, D). Staining for total p53 was mostly nuclear, with a few cells showing cytosolic staining, and was observed in the myocardial and mesenchymal layers of the embryonic heart of embryos exposed to 24-hr hypoglycemia. Similar results were obtained using rabbit polyclonal (FL-393; Santa Cruz Biotechnology, data not shown) and mouse monoclonal (DO-1; Santa Cruz Biotechnology) primary antibodies for immunohistochemistry.

Western analysis results were similar to those obtained by IHC and demonstrated a significant increase in total p53 in hearts of mouse embryos exposed to 24-hr hypoglycemia compared to controls (Fig. 18 I, J).
Immunohistochemistry demonstrated an increased phosphorylated (active) form of p53 in hearts of embryos exposed to 24-hr hypoglycemia compared to controls (Fig. 18 E, F). Control embryonic hearts expressed little or no staining for Ser-15 phosphorylated p53. The staining in hearts of hypoglycemia-exposed embryos was predominantly nuclear and was observed in myocardial and mesenchymal layers. Negative controls for total and phosphorylated p53 demonstrated no staining (not shown). Western analysis results were similar to those obtained by IHC and demonstrated a significant increase in phosphorylated (active) form of p53 in hearts of mouse embryos exposed to 24-hr hypoglycemia compared to controls (Fig. 18 K, L).

The IHC and Western analysis results for active caspase-3, total p53, and phosphorylated p53 were consistent between embryos exposed in vivo and in vitro to 24-hr hypoglycemia.

**E10.5 x 24 hr hypoglycemia.**

Total p53 protein expression in hearts of E10.5 mouse embryos exposed to 24 hr hypoglycemia was up-regulated compared to controls (Fig. 21). However the increased p53 expression in E10.5 embryonic hearts was not as dramatic as noted in E8.5 or E9.5 embryonic hearts.
Figure 17.

Immunohistochemistry of caspase-3 active subunit (p17), with fluorescently labeled secondary antibody. No increase is observed in active caspase-3 subunit staining in hearts on E8.5 mouse embryos exposed to 24 hr hypoglycemia (A) compared to controls (B). h = heart.
Figure 18.

Immunohistochemistry and Western analysis of caspase-3 active subunit (p17), total p53, and phosphorylated p53 in hearts of E9.5 mouse embryos exposed to 24 hr of hypoglycemic or control conditions. Activated caspase-3 protein expression was increased in hearts of treated vs. control embryos by immunohistochemistry (A, B) and Western analysis (G, H). Total p53 protein
expression was increased in hearts of treated vs. control embryos by immunohistochemistry (C, D) and Western analysis (I, J). p53 phosphorylated at Ser-15 residue was increased in hearts of treated vs. control embryos by immunohistochemistry (E, F) and Western analysis (K, L). Magnification = 100x, N = 3, (v) = ventricle, (a) = atria.
Figure 19.

Immunohistochemistry of caspase-3 active subunit (p17), with fluorescently labeled secondary antibody. A dramatic increase in active caspase-3 subunit staining is observed in hearts on E10.5 mouse embryos exposed to 24 hr hypoglycemia (A) compared to controls (B). Magnification = 100x, a = atria, v = ventricle.
Figure 20.

Immunohistochemistry of total p53 in E8.5 embryonic mouse hearts exposed to 24 hr hypoglycemic and control conditions. A dramatic increase in total p53 staining is observed in hearts of E8.5 mouse embryos exposed to 24 hr hypoglycemia (A) compared to controls (B). Magnification = 100x, v = ventricle.
Figure 21.

Immunohistochemistry of total p53 in E10.5 embryonic mouse hearts exposed to 24 hr hypoglycemic and control conditions. A slight increase in total p53 staining is observed in hearts of E10.5 mouse embryos exposed to 24 hr hypoglycemia (A) compared to controls (B). Magnification = 100x, a = atria, v = ventricle.
**Electron Microscopy**

Electron microscopy was performed as a preliminary study to assess whether changes in mitochondria occur as a result of hypoglycemic exposure. Hearts exposed to hypoglycemia show damaged mitochondria (Fig 22 A) as compared to intact mitochondria in control (Fig 22 B).

**PCNA**

Immunohistochemistry demonstrated minimal PCNA in heart sections of embryos exposed to 24-hr hypoglycemia compared to controls (Fig. 23 A, B). Western analysis results confirmed IHC findings and demonstrated a significant reduction in cell proliferation as measured by PCNA in the hearts of embryos exposed to 24-hr hypoglycemia compared to controls (Fig. 23 C, D). Negative controls demonstrated no staining (not shown). The PCNA results were consistent between embryos exposed in vivo and in vitro to 24-hr hypoglycemia.
Figure 22.

Electron microscopy showing structural differences in mitochondria. Arrows indicate mitochondria. **A.** Cardiac cell exposed to hyoglycemia showing disrupted mitochondria (arrow). **B.** Cardiac cell exposed to normoglycemia showing dense and compact mitochondria (arrow).
Figure 23.

Immunohistochemical PCNA staining demonstrated by brown color, which is evident in hearts of embryos exposed to control conditions (B) compared to marked reduction in hearts of embryos exposed to hypoglycemic conditions (A). C: Western analysis showing a lower density PCNA band in hearts of embryos exposed to 24-hr hypoglycemia compared to controls. D: Graphical representation of data from C. Magnification = 100x, n = 4, *p ≤ 0.05, bars = SEM, 40 = treated, 150 = control.
DISCUSSION

This study was performed in order to characterize hypoglycemia-induced cell death in embryonic mouse hearts and to evaluate potential mediators of this cell death pathway. The effects of hypoglycemia on cell death was analyzed in E8.5, E9.5 and E10.5 mouse heart, although an emphasis was placed on E9.5 heart. Glucose is the major energy source for the embryo and the embryonic heart during the organogenesis stage of development (Cox and Gunberg, 1972b). At E9.5 glucose is metabolized primarily by glycolysis because the TCA cycle is not fully functional at this stage (Kohler and Peters, 1970). Thus, glucose deficiency deprives the embryo of its major energy source for growth and development.

Exposure of the embryo to maternal diabetes during the critical period of fusion, folding, and septation is thought to produce a variety of congenital defects, including malformations of the heart (Mills et al., 1979). Brief exposures to hypoglycemia during E8.5 to E10.5 alter the embryonic heart morphologically, functionally, as well as metabolically (Smoak, 1997). Changes in cell death and cell proliferation patterns in the embryonic heart during the critical period of organogenesis may contribute to congenital heart malformations (Fisher et al., 2000).
The decreased size and function in hearts of embryos exposed to hypoglycemia compared to controls may be due to susceptibility of the embryonic heart to hypoglycemia-induced increased cell death and decreased cell proliferation. Our observations on E18.5 suggest that 24 hr of maternal hypoglycemia between E9.5 and E10.5 interferes with normal embryonic growth and survival. Embryonic glucose levels were not measured in this study but are expected to reflect maternal glycemia, considering that the placenta is readily permeable to glucose. Although the majority of embryos were viable at the end of the 24-hr exposure period, the amount of resorptions observed on E18.5 suggest that most embryos died shortly after the treatment period. The effects of 24-hr hypoglycemia on the developing heart may be the primary reason for embryonic death. Future studies will define how soon after hypoglycemic exposure these embryonic effects are manifested.

The embryonic heart has previously demonstrated resistance to apoptosis in response to several teratogenic insults, and the myocardium is particularly resistant to apoptosis (Gao et al., 1994; Mirkes and Little, 1998; Poelmann et al., 2000), although the reasons for this resistance are not known. Studies have reported cell death in cultured neonatal cardiomyocytes in response to insults, such as glucose deprivation, hypoxia, and ischemia, through activation of the mitochondrial cell death pathway (Bialik et al., 1999; Malhotra and Brosius, 1999; Moley and Mueckler, 2000). Additional mechanisms proposed previously for cell
death due to glucose deprivation include the JNK/MAPK signaling pathway, and stabilization of p53 through upregulation of HIF-1_ (Moley and Mueckler, 2000). In the present study we demonstrated that hypoglycemia induces cell death and decreases cell proliferation in the embryonic heart. We also demonstrated an increase in active caspase-3, and both total and phosphorylated p53 protein expression, and thus a potential role for these factors in hypoglycemia-induced increased cell death and decreased cell proliferation in the embryonic heart.

TUNEL is commonly used as an assay to detect cell death by labeling the 3’-OH end of nicked DNA fragments. Studies in E9 mouse embryos demonstrate co-localization of TUNEL and caspase-3 in limb-bud cells (Umpierre et al., 2001). The E8.5 embryonic heart seems to be relatively resistant to cell death induced by hypoglycemia as shown by TUNEL and Lysotracker® staining. Although no increase was observed in active caspase-3 expression in the embryonic heart in response to 24 hr hypoglycemia, there was a dramatic increase in p53 expression. Hence it may be possible that at E8.5, 24 hr hypoglycemia has a more dramatic effect on decreasing cell proliferation by up-regulating p53 expression, with cell death pathways not being activated by hypoglycemia at E8.5.

CardioTACS is a colorimetric TUNEL method modified to selectively enhance staining of cardiac cells. Both fluorescent and colorimetric TUNEL methods used in this study demonstrated an increase in DNA fragmentation and
provided clear evidence of cell death in E9.5 embryonic hearts exposed for 24 hr to hypoglycemia in vivo or in vitro. Similar results using TUNEL assay were noted in E10.5 embryonic heart exposed to 24 hr hypoglycemia.

Lysotracker detects cell death by staining lysosomes of neighboring cells that have engulfed apoptotic bodies. Based on our results with TUNEL and Lysotracker assays, we have confirmed that a 24-hr in vivo or in vitro exposure to hypoglycemia on E9.5 induces cell death in the embryonic heart. The TUNEL methods and Lysotracker assay do not distinguish between different types of cell death, but the presence of active caspase-3 subunit in the dying cells suggests that the cells are dying by apoptosis. Further studies will evaluate this phenomenon by examining other factors that may be involved in the apoptotic pathway. The importance of this work is the demonstration that hypoglycemia induces cell death in the embryonic heart at the E9.5–E10.5 stage, and that this may contribute to embryonic death or congenital heart malformations.

TUNEL and Lysotracker both demonstrated intense staining for cell death in the proepicardial organ. The proepicardium consists of migrating populations of progenitor cells, which contribute to the formation of the epicardium, coronary artery, atrio-ventricular cushions, and the myocardium (Vrancken Peeters et al., 1995; Mikawa and Gourdie, 1996; Gittenberger-de Groot et al., 1998). Excessive cell death in the proepicardial organ may contribute to abnormal formation of
these structures and may thus potentially produce congenital heart defects similar to those in the offspring of diabetic mothers.

Flow cytometry allows one to separate and isolate differentially-stained cells, and can thus be used to quantify cells undergoing cell death in the embryonic heart. A higher percentage of total cardiac cells were TUNEL-positive after exposure of E9.5 embryos to hypoglycemia compared to controls, suggesting increased overall cell death in the embryonic heart in response to 24-hr hypoglycemia. Dual staining with TUNEL and MHC demonstrated a significantly higher percentage of TUNEL-positive/MHC-positive cells in the hearts of embryos exposed to hypoglycemia compared to controls, demonstrating that cardiomyocytes are susceptible to hypoglycemia-induced cell death. There was no statistical difference in cell death between cardiomyocytes and other cardiac cells in response to hypoglycemia. Our flow cytometry results are consistent with our TUNEL, Lysotracker® and IHC findings and suggest that heart cells (cardiomyocytes and others) are susceptible to hypoglycemia-induced cell death. However the flow cytometry results also display significant levels of background fluorescence in normoglycemic cells, despite gating of fluorescent cells. Since the background fluorescence was also quantified as apoptotic cells in our experiments, our results show over 70% cell death due to hypoglycemic exposure and over 50% in normoglycemic exposures. This is not consistent with our IHC findings, and quantitation of IHC cell death by cell counting in stained
sections. The IHC data are likely to be a more accurate estimate of the amount of cell death in the embryonic heart, when compared to flow cytometry results, since it is possible to distinguish fluorescent vs. weakly or non-fluorescent cells by light microscopy. Thus this susceptibility to cell death may be important in the pathogenesis of hypoglycemia-induced congenital cardiac malformations.

Caspase-3 is cleaved in the apoptotic pathway and is a downstream effector caspase. Its presence in the active form is indicative of a cell’s fate to undergo apoptosis. Positive staining for active caspase-3 subunit in our treated embryos suggests a possible role for caspase-3 in the pathway leading to hypoglycemia-induced apoptosis. Blocking, or absence of, caspase-3 activity in the embryo produces dramatic reduction in cell death in the brain, but not in the limbs (Lee et al., 1999; Umpierre et al., 2001). These studies demonstrate the importance of caspase-3 in normal developmental apoptosis. Studies using specific inhibitors to block the activity of active caspase-3 will be needed to definitively prove its role in hypoglycemia-induced cell death in the embryonic heart.

PCNA has been widely used during the last two decades to demonstrate cell proliferation. E9.5 embryonic hearts from embryos exposed in vivo or in vitro to 24-hr hypoglycemia were evaluated for changes in cell proliferation using PCNA staining by IHC and Western analysis. Both methods demonstrated decreased cell proliferation in the embryonic heart exposed to hypoglycemia.
when compared to normoglycemia. Decreased cell proliferation may be an early response of the embryo to hypoglycemic stress, and, considering the role of p53 in cell cycle regulation, p53 may be involved in this response. There has not been consistent demonstration of p53 expression in the embryonic heart. There is no immunohistochemical detection of p53 protein in hearts of gd10 rat embryos exposed to normal or hyperthermic conditions in vitro, despite its induction in developing brain, eye, and somites (Dugyala et al., 2002). Chemically-induced genotoxic stress in gd8 mouse embryos likewise induces p53 gene expression and apoptosis in neural tissues, but not heart (Wubah et al., 1996). However, several studies have demonstrated p53 protein in hearts of normal organogenesis-stage chick embryos (Krinka et al., 2001) and human embryos (Miosge et al., 1997; Lichnovsky et al., 1998). p53 is expressed in myocardial, epicardial, and endocardial layers of the human embryonic heart (Miosge et al., 1997), and its concentration diminishes as development progresses (Lichnovsky et al., 1998). p53 mRNA is found in hearts of normal organogenesis-stage chick embryos (Krinka et al., 2001) and rat fetuses (Rees et al., 1999). Hypoxia induces p53 protein expression in primary chick cardiomyocytes (Chandel et al., 2000). The increased p53 expression demonstrated here in the embryonic mouse heart in response to 24-hr hypoglycemia coincides with decreased cell proliferation. Both total and phosphorylated p53 staining in this study are predominantly nuclear. Since p53
protein acts as a transcription factor for several other genes, including p21, its nuclear localization in the embryonic heart likely contributes to a decrease in cell proliferation.

The E10.5 embryonic heart demonstrates increased cell death induced by 24 hr hypoglycemia. TUNEL and Lysotracker® results demonstrate a significant increase in cell death, indicating the increased susceptibility of the embryonic heart to hypoglycemic insult. A dramatic difference is seen in active caspase-3 subunit staining. Increased active caspase-3 staining in the E10.5 embryonic heart exposed to hypoglycemia may indicate the involvement of caspase-3 in the mechanism leading to cell death. There is also a slight increase in p53 expression at E10.5 but the increase is not as dramatic as seen in E8.5 or E9.5 in response to 24 hr hypoglycemia.

The results suggest that the embryonic heart demonstrates increased susceptibility to decreased cell proliferation at E8.5 and increased cell death at E10.5 from exposure to 24 hr hypoglycemia. These effects may be mediated by p53 and caspase-3. These results may indicate that as the embryonic heart gets older in development is more susceptible to teratogenic insults than in earlier stages. This study helps us understand the effects of hypoglycemia at different critical stages in embryonic heart development.

It is not known what pathways are involved in hypoglycemia-mediated cell death in the embryonic heart. Hypoglycemia may cause DNA damage, which
activates p53 expression and triggers a response to inhibit cell proliferation. Our preliminary work has demonstrated decreased cell proliferation in the embryonic heart as early as 6 hr after hypoglycemic exposure. Prolonged hypoglycemic insult may activate factors that trigger the apoptotic pathway, ultimately causing the damaged cells to die. An increase in p53 protein expression observed in response to hypoglycemia may be due to a hypoglycemia-induced increase in HIF-1α (Carmeliet et al., 1998; Williams et al., 2002). HIF-1α protects p53 against Mdm2-mediated export to the cytoplasm and degradation (Chen et al., 2003). My preliminary studies have demonstrated an increase in HIF-1α and Bax expression in the embryonic hearts exposed to 24-hr hypoglycemia, using both IHC and Western analysis. Thus, hypoglycemia may induce a catalytic cascade involving HIF-1α mediated increase in Bax expression causing activation of the mitochondrial apoptotic pathway.

In this study we have demonstrated that 24-hr hypoglycemia during organogenesis interferes with growth and survival of embryos. In general, these results are consistent between 24-hr in vivo and in vitro exposures to hypoglycemia, suggesting that the in vitro culture system mimics the glycemic environment and the embryonic effects produced in vivo. We have also confirmed that hypoglycemia induces cell death in the embryonic heart using a variety of techniques, although it is difficult to clearly differentiate between apoptosis and necrosis in this study. The cell death observed is most likely
apoptotic in nature due to the presence of active caspase-3 subunit in the embryonic heart exposed to hypoglycemia, and future studies will be required towards confirming the involvement of active caspase-3 and other factors acting upstream and downstream in the pathway leading to programmed cell death. PCNA staining demonstrates the negative effect induced by hypoglycemia on cell proliferation, and the increase in p53 expression may be linked to this phenomenon. p53 needs to be further evaluated as a potential inhibitor of cell proliferation by using specific p53 inhibitors. It is likely that hypoglycemia-induced cell death and decreased cell proliferation in the embryonic heart contributes to our observed cardiac defects and decreased survival of diabetic offspring.
CHAPTER 3

Anti-Apoptotic Effects of hsp70 In Cultured Embryonic Mouse Hearts

Abstract

Heat shock protein 70 (Hsp70) is a major inducible stress protein and has long been thought to contribute to cell survival following potentially lethal stresses. Hypoglycemia induces increased cell death in E9.5 embryonic hearts as compared to normoglycemia. This study investigates the protective effect of Hsp70 against cell death in the embryonic heart exposed to 24 hr hypoglycemia. Embryonic hearts were isolated on E9.5 and injected with hsp70 over expression vector (pCMV-hsp70-hrGFP) or ‘empty’ vector (pCMV-hrGFP). The hearts were electroporated after injection and placed in hypoglycemic (40 mg/dl glucose) or normoglycemic (150 mg/dl glucose) culture medium for 24 hr. Hearts were stained with Lysotracker® and imaged using a confocal microscope or pooled and evaluated for Hsp70 and active caspase-3 expression. GFP expression was strong in all hearts, indicating successful transfection. Hearts transfected with empty vector had strong lysotracker staining after hypoglycemic exposure and this was significantly reduced in hearts transfected with Hsp70 over expression vector. Western analysis demonstrated increased Hsp70 and decreased active
caspase-3 expression in hearts injected with pCMV-hsp70-hrGFP vector. These results suggest that Hsp70 may play a protective role in embryonic hearts exposed to hypoglycemia. Over expression of Hsp70 may decrease the formation of active caspase-3 subunit, the executor of apoptosis in most cell types.
Introduction

Cells respond to external stresses, such as metabolic disturbances and injuries, in a very typical manner. The cell mounts a stress response that incorporates the induction of a number of genes encoding proteins which are induced in an effort to save the cell from death. Among these proteins are the heat shock proteins (so-called stress proteins). Heat shock protein 70 (HSP70) is the major inducible stress protein and has long been thought to contribute to cell survival following potentially lethal stresses.

Hypoglycemia is a very common clinical condition women pregnant women suffering from diabetes, particularly seen as a side effect of therapy (Nilsson et al., 1988). It is also noted in certain disease states such as starvation and tumors (Senior and Sadeghi-Nejad, 1989). Previous work in this laboratory has demonstrated that hypoglycemia affects the morphology, function and metabolism of the embryonic heart (Smoak, 1997; Ghatnekar et al., 2004). In addition, our recent work has also shown that hypoglycemia induces decreased cell proliferation and increased cell death in the embryonic heart in response to 24-hr hypoglycemia, in vivo and in vitro (Ghatnekar et al., 2004). Apoptosis has been demonstrated in both acute and chronic heart diseases (Kang, 2000). Inhibition of apoptosis is now being studied as a potential therapeutic tool for
several cardiovascular diseases (Koseki et al., 1998; Ohno et al., 1998; Zheng et al., 1999; Kang and Izumo, 2004).

The heat shock response, first observed in 1962 (Ritossa, 1962), has been widely recognized as having a significant role in cellular protection (Kiang & Tsokos, 1998), and has emerged as a primary mediator of cardioprotection due to preconditioning (Latchman, 2001). Hsp’s act as molecular chaperones and help maintain correct folding of newly synthesized polypeptides (Beckmann et al., 1990). In addition to important functions in protein refolding and transport, the Hsp70 family is also capable of binding and sequestering activated caspases, APAF and AIF making Hsps desirable targets for a role in ischemia tolerance (Jaattela et al., 1998; Beere et al., 2000; Ravati et al., 2000; Saleh et al., 2000).

Over expression of Hsc 70 (constitutive form) and Hsp70 (inducible form) protects cells from heat shock-induced cell death by preventing the processing of procaspases 9 and 3 (Mosser et al., 2000). Increased demand for the chaperoning function of Hsp70 occurs at different stages in a cell’s life, as seen by changes in its level of expression during development and through the cell cycle. Stress-induced proteins like Hsp70 may also help determine the fate of cells faced with death (Landry et al., 1989; Li et al., 1991).

Cardiac myocytes are terminally differentiated and have little potential for division; hence prevention of cell death is crucial in treatment of cardiovascular disease (Ohno et al., 1998; Kang and Izumo, 2003). Several studies have
demonstrated the expression of Hsp70 in the embryonic heart (Mirkes et al., 1999; Abdelwahid et al., 2001). Hsp70 is the most abundantly induced Hsp in a variety of stress inducing scenarios. Hsp70 has been shown to protect embryonic day 8 mouse embryos by inducing thermotolerance (Mirkes et al., 1999), and transfection of cells with Hsp70 gene demonstrated enhanced thermotolerance (Angelidis et al., 1991; Williams et al., 1993). Consistent with this function, microinjection of antibodies against Hsp70 into cells blocked the induction of thermotolerance (Riabowol et al., 1988). Hsp70 over-expressing transgenic mice have demonstrated that expression of Hsp70 increases the resistance of adult heart to ischemic injury (Marber et al., 1995; Plumier et al., 1995; Radford et al., 1996). The activity of the HSP70 promoter can be induced by moderate hyperthermia (39 °C to 43 °C) (Huang et al., 2000), reaching expression levels similar to those of the CMV promoter.

This study was undertaken to examine the effects of hypoglycemia exposure on Hsp70 expression in the embryonic mouse heart, and to over-express hsp70 in order to prevent or reduce cell death in response to 24 hr hypoglycemia. Hsp70 was over expressed using a pCMV-hsp70-hrGFP vector that was microinjected and electroporated into embryonic hearts. Following in vitro culture of hearts, staining with Lysotracker® red was used to assess cell death. Changes in Hsp70 and active caspase-3 expression in response to
hypoglycemia and Hsp70 over expression was also assessed by Western analysis.
Materials and Methods

Whole embryo Culture

The whole embryo culture (WEC) method was used to expose mouse embryos to hypoglycemic and control medium in vitro. Pregnant females were killed by cervical dislocation on E9.5 (20-somite stage), and embryos were prepared for WEC using an adaptation of methods originally described by New (1978). Embryos with an intact visceral yolk sac and ectoplacental cone were placed in culture medium composed of 75% rat serum and 25% Tyrode’s buffer. Serum was obtained by immediate centrifugation of blood withdrawn from the abdominal aorta of ether-anesthetized male Sprague-Dawley rats. Hypoglycemic serum was obtained by injecting rats with 50 international units (IU) insulin (Regular Iletin II Pork insulin; Eli Lilly, Indianapolis, IN) intraperitoneally 2–3 hr before blood withdrawal. Serum was heat inactivated, treated with antibiotics, and stored at −20°C until use. Hypoglycemic medium (40 mg/dl glucose) was prepared with glucose-free Tyrode’s buffer, and normoglycemic medium (150 mg/dl glucose) was prepared by adding concentrated (50 mg/ml) _D-glucose. Two embryos were placed in 4 ml of medium in 30 ml vials. The vials were aerated at the start of culture and at 12 hr with a gas mixture containing 20% oxygen, stoppered, and placed on a wheel rotating at 30 rpm in a 37°C incubator.
Glucose was added at 12 hr to maintain appropriate levels in hypoglycemic and control media.

**Immunohistochemistry**

Embryos removed from WEC (in vitro exposure) were fixed in 4% paraformaldehyde for 24–48 hr, paraffin embedded, sectioned sagittally at 5 μm, and placed on slides. Sections were deparaffinized in xylene, rehydrated in decreasing ethanol and 1X PBS, and permeabilized with saponin buffer (0.1% v/v in 1X PBS) for 10 min at RT. Antigen retrieval was performed with 2 N HCL at 37°C for 15 min. IHC analysis was performed for Hsp70. Negative controls for each protein were treated with 1X PBS instead of primary antibody.

**Hsp70.** Sections were stained for Hsp70 by incubating with goat polyclonal anti-Hsp70 antibody (Santa Cruz Biotechnology) at a 1:250 dilution in PBS with 2% BSA overnight at 4°C. The primary antibody is specific to Hsp70 and is cross-reactive for human and mouse Hsp70. Sections were washed in PBS and incubated with biotinylated anti-goat IgG Link (BioGenex, San Ramon, CA) for 1 hr at RT. Sections were washed in PBS and incubated with peroxidase conjugated streptavidin (BioGenex) for 30 min. 3-amino-9-ethylcarbazole (AEC) in N,N-dimethylformide (Biogenex Laboratories, San Ramon, CA) chromogen was used as a substrate for the colorimetric reaction. Sections were counterstained with hematoxylin, coverslipped using GVA (Zymed, San Francisco, CA) mounting medium, and imaged with a light microscope.
**Isolated heart culture**

Pregnant females were killed by cervical dislocation on E9.5. Hearts were isolated from embryos by severing inflow and outflow tracts and exposed to hypoglycemic and control medium. The culture medium was composed of 75% rat serum and 25% Tyrode's buffer. Serum was obtained by immediate centrifugation of blood withdrawn from the abdominal aorta of ether-anesthetized male Sprague-Dawley rats. Hypoglycemic serum was obtained by injecting rats with 50 IU insulin (Regular Iletin® II Pork insulin, Eli Lilly and Company, IN) intraperitoneally 2-3 hr before blood withdrawal. Serum was heat inactivated, treated with antibiotics, and stored at −20°C until use. Hypoglycemic medium (40 mg/dl glucose) was prepared with glucose-free Tyrode’s buffer, and normoglycemic medium (150 mg/dl glucose) was prepared by adding concentrated (50 mg/ml) β-D-glucose. Ten isolated hearts were placed in 4 ml medium in 10 ml vials. The vials were aerated at the start of culture and at 12 hr with a gas mixture containing 20% oxygen, stoppered, and placed on a wheel rotating at 30 rpm in a 37°C incubator. Glucose was added if required at 12 hr to maintain appropriate levels in hypoglycemic and control media.

**Hsp70 construct preparation**

Hsp70 cDNA was obtained as a gift from Dr. Jill A. Barnes at NC State, College of Veterinary Medicine, Raleigh, NC. The 2.0kb Hsp70 cDNA fragment
was ligated into the multiple cloning site (MCS) of the pIRES-hrGFP-1a vector (Stratagene, USA), which has a pCMV promoter upstream of the MCS region, and a hrGFP reporter gene downstream. The vector was sequenced to confirm the identity of Hsp70 (Duke University sequencing facility, Durham, NC).

**Primary heart cell culture and transfection**

Hearts were collected from normal E9.5 embryos and primary cell cultures were obtained and transfected with Hsp70 construct to confirm vector functionality. Heart cell cultures were transfected using Fugene6 and visualized for hrGFP expression using a fluorescent microscope.

**Nano-injecting the embryonic heart**

The hearts were given one hour to settle down and resume regular beating in culture medium. The vials were then removed from the incubator and hearts were placed in cold Tyrode’s buffer, in order to temporarily slow down the regular rhythm, and prepare hearts for injection. Hearts were divided into 3 groups: one group was injected with empty (pCMV-hrGFP) vector, and placed in normoglycemic medium, a second group of hearts was injected with the empty vector (pCMV-hrGFP), and placed in hypoglycemic medium, a third group of hearts was injected with the hsp70 over expression vector (pCMV-hsp70-hrGFP). Hearts were then electroporated at 20V. The hearts were placed back in culture
medium immediately after injection and electroporation for 24 hr. Post culture the hearts were stained with Lysotracker® or collected and stored for Western analysis.

**Lysotracker Staining**

Lysotracker is a vital stain for lysosomes that, because of their acidity, become more apparent after engulfing apoptotic bodies. Hearts were stained according to previously described methods (Zucker et al., 1999). Hearts were removed from culture medium, and placed in 5 mM Lysotracker in Hank’s balanced salt solution (HBSS) for 30 min at 37°C. Hearts were rinsed in cold 1X PBS and fixed in 4% paraformaldehyde for 24 hr in the dark at 4°C. Hearts were dehydrated with methanol and cleared with 1:1 benzyl alcohol and benzyl benzoate (BABB) for 1 hr. Hearts were placed in BABB in depression slides, coverslipped, and imaged using a Nikon C-1 confocal microscope (Nikon Instruments Inc., Melville, NY).

**Western analysis**

Isolated hearts post culture were sonicated in sonication buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA), and analyzed for total protein concentration using the Bradford (1976) method and BSA standards. Proteins (20 µg per sample) were resolved on a 4–15% gradient gel at 200 volts and
transferred to a polyvinylidene fluoride (PVDF) (Bio-Rad Laboratories, Hercules, CA) membrane at 100 volts for 1 hr. The membrane was blocked with 5% non-fat dry milk in TTBS for 1 hr, and probed with primary antibody. The membrane was washed in TTBS and exposed to peroxidase labeled secondary antibody (Amersham International Plc, Amersham, UK) for 1 hr. The membrane was washed and exposed to chemiluminescence substrate ECL (Amersham Pharmacia Biotech UK, Buckinghamshire, UK). Membranes were stripped and re-probed for actin as a loading control. Membranes were imaged using a UVP Epi Chemi II darkroom (UVP Products, Upland, CA) or wrapped in plastic and exposed using x-ray film. Bands were scanned and densities quantified as a ratio of target protein to actin.

**Hsp70.** Membranes were probed using 1:100 concentration of anti-Hsp70 goat polyclonal primary antibody (Santa Cruz Biotechnology) overnight at 4°C and 1:10,000 rabbit anti-goat secondary antibody for 1 hr at RT.

**Active caspase-3.** Membranes were probed using 1:1000 concentration of anti-active caspase-3 rabbit polyclonal primary antibody (Promega) overnight at 4°C and 1:100,000 goat anti-rabbit secondary antibody for 1 hr at RT.
Data Analysis

Numerical data were statistically analyzed with Student’s t-test (Statistical research methods in the life sciences by P.V. Rao). A significance level of $p < 0.05$ was maintained throughout.
Results

**Immunohistochemistry**

Immunohistochemistry was performed to assess normal levels of Hsp70 expression in embryonic mouse heart. IHC demonstrated no difference in Hsp70 protein expression in hearts of embryos exposed to 24-hr hypoglycemia compared to controls (Fig. 24). The staining for Hsp70 was evenly distributed throughout the embryonic heart in both treated and control conditions.

**Transfection**

Since Hsp70 protein levels appear to be similar in developing mouse heart under both hypoglycemic and normoglycemic conditions, an Hsp70 expression plasmid was prepared in pCMV-pIRES-hrGFP mammalian expression plasmid. This plasmid allows bicistronic expression of Hsp70 and hrGFP under the control of the CMV promoter. Thus, transfection with this plasmid allows identifying Hsp70 expressing cells by virtue of hrGFP expression. Primary heart cell cultures were successfully transfected with the Hsp70 over expressing construct based on expression of hrGFP protein (Fig 25). Transiently transfection efficiency was approximately 5%. Transfected cells demonstrated expression of hrGFP, indicating a functional pCMV-hsp70-hrGFP construct.
Figure 24.

Immunohistochemistry for Hsp70 in E9.5 embryonic mouse hearts exposed to 24 hr hypoglycemic and control conditions. No significant difference in Hsp70 staining is observed in hearts of E9.5 mouse embryos exposed to 24 hr hypoglycemia (A) compared to controls (B). Magnification = 100x, a = atria, v = ventricle.
Primary cardiac cell culture transiently transfected with pCMV-hsp70-hrGFP over expression vector. The cells transfected successfully express hrGFP and fluoresce green. This indicates that the construct created is functional.
**Lysotracker® staining**

To assess whether Hsp70 expression is crucial to counteracting hypoglycemia-induced cell death, transfected embryonic hearts were stained with Lysotracker® red in order to assess cell death.

My first analysis focused on hearts injected with empty (pCMV-hrGFP) vector and exposed to normoglycemic medium demonstrated no Lysotracker® red staining. However hrGFP expression was evident in heart cells which were transfected and electroporated, indicating that intact hearts can be successfully transfected and can express the exogenous gene from the transfection plasmid (Fig 26 A).

My next analysis focused on hearts injected with empty (pCMV-hrGFP) vector and exposed to hypoglycemia, and then stained with Lysotracker® red. These hearts, after exposure to Lysotracker®, demonstrated dual staining, staining orange due to green staining from hrGFP combined with red stain from lysotracker staining. The dual stained cells are indicative of cell death. Most transfected cells in these hearts show susceptibility to cell death due to hypoglycemic exposure (Fig 26 B). However, in figure 26 B there are some cells which only stain red since these cells were not transfected with the empty vector. These cells are shown by an arrow.

In order to investigate how Hsp70 may contribute to prevention of cell death in hypoglycemic hearts, hearts were injected with the hsp70 over
expression vector (pCMV-hsp70-hrGFP) and then stained with Lysotracker® red stain. Although hrGFP expression was evident in heart cells which were successfully transfected after electroporation (Fig 26 C), no red staining was observed, which is suggestive of no cell death in hearts over expressing Hsp70.
Figure 26.

A. Heart injected with pCMV-hrGFP vector, exposed to 24 hr normoglycemia (control), and stained with Lysotracker® red. Heart demonstrates green staining indicative of hrGFP expression. No Lysotracker® red staining is indicative of no cell death.

B. Hearts injected with pCMV-hrGFP vector, exposed to 24 hr hypoglycemia, and stained with Lysotracker® red. Hearts demonstrate dual staining for hrGFP and Lysotracker®, indicating hearts undergoing cell death due to exposure to hypoglycemia. Green staining from hrGFP and red from Lysotracker® gives orange color to stained cells. Arrows indicate cells stained with Lysotracker® red only since they did not take up the hrGFP expressing vector.
C. Heart injected with pCMV-hsp70-hrGFP over expression vector, exposed to 24 hr hypoglycemia, and stained with Lysotracker® red. Absence of Lysotracker® red is suggestive of no cell death. Hearts demonstrate green hrGFp expression. Note: Heart appears to be smaller in size compared to panel A. Magnification 400X.
**Western analysis**

Since transfection and over expression of cultured embryonic mouse hearts exhibit pronounced hrGFP expression, and decreased apoptosis under hypoglycemic conditions following transfection with pCMV-Hsp70-hrGFP, it is critical to confirm that Hsp70 was over expressed in transfected hearts. In addition, I wanted to assess whether pro-apoptotic protein, such as active caspase-3, is reduced in hypoglycemic hearts as a result of Hsp70 expression.

Western analysis demonstrated no significant difference in Hsp70 expression between the hearts injected with empty (pCMV-hrGFP) vector and exposed to hypoglycemia, and hearts injected with empty (pCMV-hrGFP) vector and exposed to normoglycemia. However, increased Hsp70 protein expression was observed in the hearts injected with the hsp70 over expression vector (pCMV-hsp70-hrGFP) and exposed to 24 hr hypoglycemia. (Fig 27 A, D).

To assess the effect of Hsp70 protein levels on active caspase-3 expression in embryonic mouse heart, western analysis for active caspase-3 was conducted. These data demonstrated a significant increase in active caspase-3 expression in hearts injected with empty (pCMV-hrGFP) vector and exposed to hypoglycemia, as compared to hearts injected with empty (pCMV-hrGFP) vector and exposed to normoglycemia (Fig 27 B, E). Hearts injected with the hsp70 over expression vector (pCMV-hsp70-hrGFP) and exposed to 24 hr hypoglycemia demonstrated a significant decrease in active caspase-3 expression.
<table>
<thead>
<tr>
<th>Lanes</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCMV-Hsp70</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Hypoglycemia</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

**Panel A.** Hsp70 70kDa

**Panel B.** Active caspase-3 17kDa

**Panel C.** Actin 45kDa

<table>
<thead>
<tr>
<th>40</th>
<th>150</th>
<th>40(Hsp70)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypo</td>
<td>Normo</td>
<td>Hypo</td>
</tr>
</tbody>
</table>
Figure 27 (A-C).

A. Western analysis for Hsp70. Lane 1 is sample from hearts injected with pCMV-hrGFP vector and exposed to 24 hr hypoglycemia. Lane 2 is sample from hearts injected with pCMV-hrGFP vector and exposed to normoglycemia. Lane 3 is sample from hearts injected with pCMV-hsp70-hrGFP over expressing vector and exposed to hypoglycemia. Lane 3 demonstrates over expression of Hsp70 protein due to the over expressing vector.

B. Western analysis for active caspase-3 subunit. The samples in the lanes correspond to the lanes in A. Lane 1 demonstrates increased active caspase-3 expression in response to hypoglycemia compared to normoglycemia (lane 2). However lane 3 demonstrates a significant decrease in caspase-3 expression despite exposure to hypoglycemia.

C. Band showing actin as internal control. Quantification of bands was done as ratio to actin.
Quantification of Hsp70 protein levels in transfected hearts. These data demonstrate a significant increase in Hsp70 expression by Western analysis in samples of hearts injected with hsp70 over expressing vector as compared to samples of hearts injected with empty vector and exposed to hypoglycemia or normoglycemia. No significant difference in Hsp70 expression was noted between samples of hearts injected with empty vector and exposed to hypoglycemia or normoglycemia. $n = 3$, $^*p \leq 0.05$, bars = SDM, 40 = hypoglycemia, 150 = normoglycemia.
Figure 27 E.

Quantitation of active caspase-3 protein levels in transfected hearts. These data demonstrate a significant decrease in active caspase-3 expression by Western analysis in samples of hearts injected with hsp70 over expressing vector, as compared to samples of hearts injected with empty vector and exposed to hypoglycemia. No significant difference in active caspase-3 expression was noted between samples of hearts injected with empty vector,
exposed to normoglycemia, and hearts injected with hsp70 over expressing vector exposed to hypoglycemia. n = 3 experiments, *p ≤ 0.05, bars = SDM 40 = hypoglycemia, 150 = normoglycemia.
Discussion

The Hsp70 gene encodes a major stress-inducible heat shock protein (HSP70) that plays an important role in protecting cells from deleterious stresses. Hsp proteins are highly conserved, abundantly expressed proteins with diverse functions (Welch, 1993). Hsp70 along with Hsp25 have been shown to play a role in development, differentiation, and apoptosis (Moreau et al., 1998; Sapozhnikov et al., 1999; Park et al., 2000). The heat shock response of the cell results in the induction of the synthesis of Hsp’s. These bind to damaged proteins and allow them to refold into their proper shapes. The induction of stress response is associated with, but not necessary for; suppression of apoptosis (Cox et al., 1994).

Induction of glucose regulated stress proteins (GR-HSP) genes during stress is regulated primarily at the transcriptional level. Evidence suggests that different stress stimuli use distinct signaling pathways to activate GR-HSP genes, and the activation patterns are tissue specific (Pahl & Baauerle, 1995; Lee, 2001; Patil & Walter, 2001). Hypoglycemia induces increased cell death in the embryonic heart during the critical period of organogenesis, contributing to congenital heart malformations (Ghatnekar et al., 2004). Hsps have been shown to protect against apoptotic cell death triggered by a variety of stimuli (Garrido et al., 1999), and have been implicated in preconditioning, decreasing ROS
production and the blockade of cell death after cytochrome c release (Mehlen et al., 1996; Garrido et al., 1999; Cohen et al., 2000). Despite these effects, my initial experiments using E9.5 embryos exposed to 24 hr hypoglycemia in vitro using, whole embryo culture, I observed no upregulation of Hsp70 in response to hypoglycemia. This may be due to a tissue specific activation pattern of stress proteins.

Cells transfected with Hsp70 gene demonstrate enhanced thermotolerance (Angelidis et al., 1991; Williams et al., 1993). Since my preliminary studies did not demonstrate up-regulation of Hsp70 protein expression in response to hypoglycemia, this study demonstrates the effects of over expressing Hsp70 in E9.5 embryonic isolated heart cultures in response to 24 hr hypoglycemia. The isolated heart culture method was used since it is difficult for whole embryos to survive for 24 hr under hypoglycemic stress, and post- electroporation. However isolated hearts are still alive and beating post-electroporation and in the isolated culture. Hsp70 over expression was achieved by injecting isolated hearts with the desired construct, and hearts were electroporated to facilitate uptake of foreign DNA by the heart cells. Post-culture the hearts were stained with Lysotracker® red in order to visualize cell death. Significant cell death was observed in hearts exposed to hypoglycemia after injection with the empty control vector. However no cell death was observed in hearts injected with the empty control vector and exposed to normoglycemia.
These results were consistent with our previous findings (Ghatnekar et al., 2004), and indicate that hypoglycemia, in the absence of an up-regulation of Hsp70, leads to enhanced cell death. However, the hearts injected with the Hsp70 over expressing construct, and exposed to 24 hr hypoglycemia, did not stain with Lysotracker red, suggesting that Hsp70 over expression actually protected the heart against hypoglycemia-induced cell death. These data are important when viewed of the absence of Hsp70 up-regulation by hypoglycemia in vivo, as an ability to induce hsp70 may serve as a protective mechanism for hypoglycemic cardiac tissue. This is accord with the other studies that show similar protective effects of Hsp70.

Hsp70 is known to bind to active caspase-3 and also other upstream cell death activators such as caspase-9 and cytochrome c (Beere et al., 2000; McLaughlin et al., 2003). It may be possible that one mechanism by which Hsp70 over expression in the embryonic heart prevents cell death is by impairing the ability of active caspase-3 to activate its downstream effectors of cell death. This argument is supported by my western analysis for active caspase-3, which demonstrates that expression patterns of active caspase-3 protein in embryonic hearts, injected with Hsp70 over expression vector and exposed to hypoglycemia, are reduced when compared to hearts injected with the empty control vector, exposed to normoglycemia or hypoglycemia. The hearts injected with empty vector and exposed to hypoglycemia demonstrated significantly
increased levels of activated caspase-3 as compared to hearts exposed to normoglycemia, which is likely to contribute to the enhanced cell death that is observed. However, the hearts injected with Hsp70 over expressing vector demonstrated a significant decrease in active caspase-3 expression and decreased cell death. The levels of active caspase-3 expression in the hearts injected with Hsp70 over expressing vector were almost identical to active caspase-3 expression in the hearts injected with empty vector and exposed to normoglycemia. Thus, there is a strong correlation between Hsp70 levels, active caspase-3, and cell death in my cultured isolated hearts.

Further studies will need to be conducted to evaluate the effects of Hsp70 over expression in embryonic hearts exposed to hypoglycemia, on other pro-cell death factors such as caspase-9 and cytochrome c. To date experimental evidence shows that Hsps are unique in their ability to contribute to multiple regulatory signaling cascades (Beere et al., 2000; Abdelwahid et al., 2001; McLaughlin et al., 2003). Prevention of unnecessary apoptosis may help prevent malformations in the long run (Nicholson, 2000). This makes Hsps intriguing candidates for gene therapy in conditions such as hypoglycemia. A deeper understanding of their regulation on a genetic level, and of the mechanisms through which they function in multiple regulatory circuits, will provide insight into their application in prophylaxis and therapy.
GENERAL DISCUSSION

Approximately 18% of pregnancies terminate by abortion or miscarriage, and perinatal mortality in diabetic women without proper therapy is very high. There is increasing evidence that congenital cardiac malformations seen in diabetic offspring may be the result of hypoglycemic insult during the critical period of cardiac development (Buchanan et al., 1986; Akazawa et al., 1987; Sadler & Hunter, 1987). Hypoglycemia is a common condition as a result of insulin injections commonly taken by diabetic women (Nilsson et al., 1988; Smoak, 1997). Diabetes causes a delay in pancreatic alpha cell response, which causes a brief period of hypoglycemic state after insulin administration to control high blood glucose levels. The growing embryo is exposed to the hypoglycemic state along with the mother, and the developmental period and length of this insult determine the occurrence and extent of congenital malformations in the offspring.

In normal cardiac development, the formation of the cardiac septa occurs in connection with the development of shape of the external cardiac wall. Changes in form of the external cardiac wall precede changes in the internal cardiac wall. The originally straight tubular heart is angulated at typical sites during cardiac looping, and septation and valvulogenesis follow cardiac looping (Noden & DeLahunta, 1985; Smoak, 2000). The anlagen of the cardiac septa are
not isolated formations, but rather represent a continuous system of protrusions (Steding & Seidi, 1980). This major period of cardiac development takes place in the rather critical period of organogenesis in the early embryo. In the mouse embryo this development occurs mostly between E8.5 and E11.5. Similar development in human embryos takes place during the end of the first month of pregnancy.

Hypoglycemic insult to the embryo during this critical period of cardiac development can lead to formation of congenital cardiac defects. A study by Steding and Seidi in 1981 explains the various scenarios that can lead to congenital cardiac malformations. The organogenesis stage embryonic heart, along with the early embryo, is heavily dependent on glycolysis, as the TCA cycle is not completely functional in early embryonic development (Tanimura & Shepard, 1970; Clough & Wittingham, 1983). Hence, lack of glucose to cardiac cells can cause serious adverse effects on early embryonic heart development (Akazawa et al., 1989; Akazawa et al., 1994). My study demonstrates that hypoglycemia is capable of inducing excessive cell death during the organogenesis stage in embryonic mouse heart. This excessive cell death during any of the critical periods in cardiac developments can lead to defects such as tetralogy of Fallot, truncus communis, aortico-pulmonary window, and rare defects such as double outlet right ventricle, and criss-cross heart (Steding & Seidi, 1981). These defects can be lethal to the survival of the offspring. Hence it
is essential to understand the mechanisms by which hypoglycemia affects embryonic heart development.

The embryonic heart shows both metabolic and morphological alterations in response to hypoglycemic exposure both in vivo and in vitro. Gross abnormalities of cardiac development lead to embryonic demise either during mid-gestation or in the peripartum period. Embryos exposed to hypoglycemia are grossly smaller in size. The function of the embryonic heart also seems to be affected as a result low energy availability. A decrease in the contractile function of the heart can also be observed as a result of low energy availability..

The embryonic heart responds to hypoglycemia or decreased glucose availability by increasing glucose uptake. This can be demonstrated using $^{13}$C-NMR spectroscopy, as shown by my studies in chapter 1. Glucose metabolites can be detected in embryonic mouse tissues using $^{13}$C-NMR spectroscopy. The advantage of this method is in its chemical specificity and the ability to follow metabolic changes. My results confirm earlier findings using radiolabeled substrates and suggest that $^{13}$C-NMR spectroscopy can be used to study glucose metabolism in isolated embryonic hearts exposed to hypoglycemia. NMR effectively measures glucose and its metabolite, lactate, in the same spectrum and thus determines metabolic flux in the isolated embryonic heart after exposure to hypoglycemia and normoglycemia. My results demonstrate increased lactate production in the embryonic hearts exposed to hypoglycemia.
Lactate is the metabolic end point in glycolysis and thus can be used as a good measure of glucose utilization and energy production through glycolysis. This method can also be used to evaluate glucose metabolism in embryonic tissues following other teratogenic exposures.

Cardiovascular disease is one of the major threats in today’s world. Adult cardiomyocytes are terminally differentiated cells and once destroyed are not replaced (Gill et al., 2002; Kang & Izumo, 2003). Cell death could be by apoptosis or necrosis. The last decade has seen several papers on apoptotic cell death in cardiomyopathies (Narula et al., 1996; Seki et al., 1998; Kang, 2000; Knaapen et al., 2001). Embryonic heart cells are largely resistant to teratogen-induced apoptosis (Mirkes et al., 2001). Although the reasons for this resistance are not known, several studies have reported cell death in cultured neonatal cardiomyocytes in response to insults, such as glucose deprivation, hypoxia, and ischemia, through activation of the mitochondrial cell death pathway. Cell death in embryonic cardiac tissue has been previously described as apoptotic based on TUNEL staining and immunohistochemical detection of active caspase-3 subunit (Knaapen et al., 2001). My work demonstrates a significant increase in hypoglycemia-induced cell death in the embryonic heart at E9.5 and E10.5. In contrast, no significant increase in cell death in response to hypoglycemia was seen at E8.5. (Ghatnekar et al., 2004) Active caspase-3 expression patterns were consistent with cell death seen in the embryonic heart. However p53
expression was more dramatic in E8.5 embryonic hearts exposed to hypoglycemia when compared to control medium, suggesting that at E8.5 the embryonic heart’s response to hypoglycemic insult is predominantly by decreasing cell proliferation. A significant decrease in cell proliferation was observed in E9.5 embryonic hearts exposed to hypoglycemia, further explaining the morphological effects observed as a result of hypoglycemic exposure. These results suggest the involvement of p53 and active caspase-3 in the mechanism by which hypoglycemia induces cell death in the embryonic heart, and reduces the size of embryonic hearts in response to hypoglycemia. Further studies involving inhibiting expression of caspase-3 or p53 at the gene or protein level may provide added insight into the involvement of these factors in hypoglycemia-induced apoptosis.

After demonstrating increased cell death in E9.5 embryonic mouse heart, I examined possible mechanisms that could be employed to prevent excessive cell death in response to hypoglycemia. Hsp70 has been indicated in several studies as a stress protein aiding in preventing cell death in tissues exposed to different insults (Lee, 2001; Oglesbee et al., 2002). Although Hsp70 expression is induced in cells in response to stress, I did not observe a significant increase in Hsp70 protein expression in embryonic hearts exposed to hypoglycemia, using WEC. These data therefore provide one possible explanation for why enhanced cell death is observed in embryonic heart in response to hypoglycemia.
Since Hsp70 was not significantly induced by hypoglycemic stress, I over-expressed Hsp70 in isolated embryonic hearts by injecting and electroporating the hearts with an Hsp70 over-expressing plasmid vector. The hearts were then exposed to hypoglycemia, and appropriate controls were conducted (such as empty vector transfections and normoglycemic medium). Hsp70 over-expression in the embryonic heart exposed to hypoglycemia significantly reduced the incidence of cell death due to hypoglycemic insult. Hsp70 has been shown to prevent the processing of caspases-3 and 9, and is also believed to inhibit cytochrome c action (Beere et al., 2000; Abdelwahid et al., 2001; McLaughlin et al., 2003). This may allow Hsp70 to prevent cell death in the embryonic hearts that were transfected with hsp70 expression plasmid. These results may therefore provide important insights into the mechanisms regulating excessive cell death in the heart due to hypoglycemia, and ultimately aid in preventing congenital cardiac malformations as a result of hypoglycemia.
Figure 2.

General overview of the apoptotic pathway in mammalian cells. The factors emphasized in this thesis are labeled in green.
As shown in figure 2, many proteins are capable of regulating the apoptotic pathway in mammalian cells, with my studies examining some of these factors such as p53, caspase-3, and Hsp70. Another level of regulation of this pathway is likely by HIF-1α (Willllams et al., 2002; Sugishita et al., 2004), which can regulate Bax expression (Carmeliet et al., 1998). HIF-1α activates Bax expression, and Bax in turn causes release of cytochrome c from mitochondria, thereby activating the mitochondrial cell death cascade (Fig 2). Direct activation of mitochondrial cell death pathway is also possible (Aoki et al., 2002).

My findings suggest that the ordinarily resistant embryonic heart to different teratogens is susceptible to cell death induced by hypoglycemia. Hsp70 over-expression helps prevent occurrence of excessive cell death in the heart induced by hypoglycemia. My findings provide a possible strategy for modulating cardiac apoptosis. Further investigation is required to clarify the complex and intricate balance between apoptosis and congenital cardiac malformations. A detailed study of these mechanisms will ultimately aid in preventing congenital malformations seen in the offspring born to diabetic mothers, and also may help to prevent embryo lethality as a result of hypoglycemia.

Although I have demonstrated that hypoglycemia induced apoptosis can result in cardiac malformations in the embryonic mouse model, it cannot be unambiguously concluded that hypoglycemia-induced apoptosis is the sole factor that contributes to cardiac defects in children from diabetic mothers. For
example, some of the defects that are seen in offspring of diabetic mothers may be due to ketosis (Robles-Valdes et al., 1976). Although this may be true in neonates it is unlikely in the early embryo because the ketone bodies probably do not cross the placental barrier.

Further studies will need to be performed to evaluate whether inhibiting HIF-1α prevents cell death in the embryonic heart, using markers such as TUNEL staining and active caspase-3 expression. Studies can also be done using Hsp70 over-expressing transgenic mice to demonstrate the effects of Hsp70 over-expression in vivo in response to hypoglycemia. It is clear however, that hypoglycemia elicits a complex molecular pathway that can regulate apoptosis on several levels.
LITERATURE CITED


Tewari M, Quan LT, O'Rourke K, Desnoyers S, Zeng Z, Beidler DR, Poirier GG, Salvesen GS, Dixit VM. (1995) Yama/CPP32 beta, a mammalian homolog of
CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. Cell 81:801-809.


