Glucose Uptake and Glycolysis Reduce Hypoxia-induced Apoptosis in Cultured Neonatal Rat Cardiac Myocytes*

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Myocardial ischemia/reperfusion is well recognized as a major cause of apoptotic or necrotic cell death. Neonatal rat cardiac myocytes are intrinsically resistant to hypoxia-induced apoptosis, suggesting a protective role of energy-generating substrates. In the present report, a model of sustained hypoxia of primary cultures of Percoll-enriched neonatal rat cardiac myocytes was used to study specifically the modulatory role of extracellular glucose and other intermediary substrates of energy metabolism (pyruvate, lactate, propionate) as well as glycolytic inhibitors (2-deoxyglucose and iodoacetate) on the induction and maintenance of apoptosis. In the absence of glucose and other substrates, hypoxia (5% CO₂ and 95% N₂) caused apoptosis in 14% of cardiac myocytes at 3 h and in 22% of cells at 6–8 h of hypoxia, as revealed by sarcolemmal membrane blebbing, nuclear fragmentation, and chromatin condensation (Hoechst staining), terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining, and DNA laddering. This was accompanied by translocation of cytochrome c from the mitochondria to the cytosol and cleavage of the death substrate poly(ADP-ribose) polymerase. Cleavage of poly(ADP-ribose) polymerase and DNA laddering were prevented by preincubation with the caspase inhibitors benzylxoycarbonyl-Val-Ala-Asp-fluoromethyl ketone (zVAD-fmk) and benzylxoycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone (zDEVD-fmk), indicating activation of caspases in the apoptotic process. The caspase inhibitor zDEVD-fmk also partially inhibited cytochrome c translocation. The presence of as little as 1 mM glucose, but not pyruvate, lactate, or propionate, before hypoxia prevented apoptosis. Inhibiting glycolysis by 2-deoxyglucose or iodoacetate, in the presence of glucose, reversed the protective effect of glucose. This study demonstrates that glycolysis of extracellular glucose, and not other metabolic pathways, protects cardiac myocytes from hypoxic injury and subsequent apoptosis.

Apoptosis, a form of cell death characterized by cell shrinkage, plasma membrane blebbing, chromatin condensation, and genomic DNA fragmentation, is essential for development, maintenance of tissue homeostasis, and elimination of harmful and diseased cells in metazoan organisms (1, 2). On the other hand, dysregulated apoptosis has been implicated in many human diseases such as cancer and neurodegenerative diseases (e.g. Alzheimer’s disease, AIDS encephalopathy, and ischemic stroke) (3–6). Recently, apoptosis of heart muscle has elicited interest because of the potential role that such apoptosis may play in the development of congestive heart failure and ischemic injury (7, 8). Cardiac myocyte apoptosis can be induced by many stimuli, including hypoxia (9), ischemia/reperfusion (10), myocardial infarction (11), mechanical stretch (12, 13), staurosporine (14), tumor necrosis factor α (15), and aortic constriction (16). Apoptosis also occurs during heart failure and may contribute to its progression (17, 18). The underlying molecular mechanisms by which these diverse stimuli trigger apoptosis in the heart cells, however, remain enigmatic.

Over the years various components of the apoptotic death machinery in eukaryotic cells have been identified, based, in part, on genetic analysis of the nematode Caenorhabditis elegans, which showed that three genes, CED-3, CED-4, and CED-9, were involved in its cell death cycle (19). All three genes have mammalian homologs. CED-3 corresponds to a family of mammalian cysteine proteases known as caspases (a total of 14, at last count) and constitute the main effector arm of the death pathway (20–22). CED-9 corresponds to the mammalian anti-apoptotic genes Bcl-2 and BclXL, which serve as inhibitors of the apoptotic process (23, 24). The mammalian homolog of CED-4 was recently discovered to be Apaf-1 (apoptosis-promoting factor-1) which, with cytochrome c, procaspase 9, and dATP, forms the so-called apoptosome that participates in transmitting the final death signal by cleaving and activating caspase 3, a major effector caspase (25).

Cardiac myocytes are postmitotic, terminally differentiated cells. Hypoxia/ischemia, with or without reoxygenation, is a potent stimulator of apoptotic death in both neonatal and adult cardiac myocytes in tissue culture and in vivo (26–28). Most previous reports on the induction of apoptosis in primary cultures of neonatal rat cardiac myocytes have documented the need for long term hypoxia of 24–72 h for the appearance of significant apoptosis (9, 29). This prolonged viability in the face of severe hypoxia, which distinguishes these cells from some other cell types (30–32), suggests the presence of intrinsic mechanisms in cardiac myocytes which protect them from hypoxia-related apoptosis. Some of this relative resistance to hypoxia may be caused by the high levels of endogenous apoptosis inhibitors in these cells, such as the cellular FLIPS (cFLIP) (cellular FLICE-inhibitory proteins) and ARC (apoptosis repressor with caspase recruitment domain), which have been reported to interact selectively with the death domain of the Fas (CD95/Apo-1) death receptor and upstream initiator caspases 2 and 8, respectively (33, 34). However, the precise role of these inhibitors in heart cells has not been documented.

Under normoxic conditions, cardiac myocytes produce most of their ATP by oxidative phosphorylation, making the regula-
tion of this process central to balancing cardiac energy metabolism (35). It is estimated that approximately 60–70% of myocardial energy is obtained from the metabolism of fatty acids, and the remaining is derived from non-lipid sources including carbohydrates, ketone bodies, and amino acids (36). This fact is evident from the observation that cardiac myocytes cultured under glucose-free normoxic conditions remain viable and beat synchronously for several days. However, during ischemia or hypoxia, glucose uptake and glycolysis become critical to the maintenance of myocardial viability. Multiple studies have suggested that glucose uptake and glycolysis can prevent cardiac myocytes from ischemic or hypoxic injury in vivo or in vitro (37–39). Hypoxia and ischemia induce increased glucose uptake in part by induction of translocation of the major glucose transporter GLUT-4 to the sarcolemma, and prolonged ischemia also increases the expression of GLUT-1 (40, 41). However, no studies have documented the role that glucose uptake and glycolysis may play either in the induction or prevention of apoptosis in the heart. Therefore, in the present study we investigated whether glucose uptake and glycolysis prevented hypoxia-induced apoptosis of neonatal rat cardiac myocytes. We also investigated the potential role of various intermediaries of energy metabolism, namely fatty acids (propionate), pyruvate, and lactate, in modulating hypoxia-induced apoptosis of cultured neonatal rat cardiac myocytes.

In the present study we demonstrate that hypoxia induced substantial apoptosis by 3–8 h in cultured neonatal rat cardiac myocytes in the absence of glucose. This process was accompanied by the translocation of cytochrome c to the cytosol in a caspase 3 activation-dependent manner. Only glucose uptake and glycolysis protected cultured neonatal rat cardiac myocytes from hypoxia-induced apoptosis. Other substrates were ineffectual.

**MATERIALS AND METHODS**

**Myocyte Cell Culture**

Primary cultures of neonatal rat cardiac myocytes were prepared by a modification of a protocol reported previously (42). Briefly, cardiac myocytes were obtained from ventricular tissue of 1-day-old Wistar rats by six to seven 15-min digestions at 37 °C in HIEPES-buffered saline solution containing 0.1% collagenase IV, 0.1% trypsin, 15 μg/ml DNase I, and 1.0% chicken serum. The dissociated cells were collected by centrifugation and resuspended in ADS buffer (in g/liter: 6.8 NaCl, 4.76 HEPES, 0.138 NaH2PO4, 0.6 glucose, 0.4 KCl, 0.205 MgSO4, 0.002 phenol red, pH 7.4). The cells were then selectively enriched by differential centrifugation through a discontinuous Percoll (Amersham Pharmacia Biotech) gradient of densities 1.050, 1.062, and 1.082 g/ml (43). The band at the 1.062/1.082 density interface was collected and used as the source of cardiac myocytes. The cardiac myocytes were washed and suspended in Dulbecco’s modified Eagle’s medium (DMEM)/F-12 medium (Life Technologies, Inc.) (1:1, v/v) supplemented with 5% horse serum, 3 mM pyruvic acid, 100 μM ascorbic acid, 1 μg/ml transferrin, 10 ng/ml selenium, and 100 μg/ml ampicillin. Cardiac myocytes were plated on gelatin-precocated 60-mm dishes at a density of 2.5 × 10⁶ cells/dish. The cells were also plated on gelatin-coated Falcon culture slides at a density of 1.5 × 10⁶ cells/cm². Bromodeoxyuridine at a final concentration of 0.1 mM was added during the first 36 h to prevent proliferation of cardiac fibroblasts. Cardiac myocyte purity was monitored by immunofluorescence after staining with monoclonal antibodies specific for cardiac α-sarcomeric actin (Sigma). Myocyte purity averaged 96 ± 3% 48 h after plating. Cardiac fibroblasts were collected from the upper density gradient of 1.050/1.062 and cultured on separate untreated 100-mm plates in DMEM supplemented with 10% fetal calf serum and antibiotics.

1 The abbreviations used are: DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; TUNEL, terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling; PARP, poly(ADP-ribose) polymerase; Z-VAD-fmk, benzoylcarbonyl-Val-Ala-Asp-fluoromethyl ketone; Z-DEVD-fmk, benzoylcarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone; Z-FA-fmk, benzoylcarbonyl-Phe-Ala-fluoromethyl ketone.
Exposure to Hypoxia

All experiments of cardiac myocytes and hypoxia were done after 4 days of plating. The cells were initially plated in DMEM/F-12 medium (1:1) which contained 17.5 mM glucose. This was replaced with glucose- and pyruvate-free DMEM for hypoxia experiments; however, serum was not removed because of a report suggesting that serum withdrawal can induce apoptosis in primary cultures of cardiac myocytes (44). For hypoxia experiments, the cells were placed in a Plexiglas chamber, and a constant stream of water-saturated 95% N₂ and 5% CO₂ was maintained over the culture. To lower the P O₂ to 5 mm Hg, Oxyrase, a mixture of bacterial membrane monooxygenases and dioxygenases (Oxyrase Inc., Ashland, OH) was added to the culture medium at a final concentration of 2–6%. As shown in Fig. 1 cardiac myocytes cultured in 6% Oxyrase for 24 h under normoxic conditions do not show any DNA laddering or morphologic signs of apoptosis.

Hoechst and TUNEL Staining

For morphological studies the cardiac myocytes were grown in eight-well gelatin-coated Falcon glass culture slides (Becton Dickinson Labware, Franklin Lakes, NJ). The cells were rinsed in phosphate-buffered saline (PBS), pH 7.4, and fixed for 30 min in 4% paraformaldehyde in PBS, pH 7.4, at room temperature. After a rinse in PBS, the cells were permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate. The cells were rinsed twice in PBS and then stained with the karyophilic dye Hoechst 33258 (5 μg/ml) for 10 min at room temperature. After a final rinse in PBS, the cells were mounted in moiwol, an antifade agent, and visualized under ultraviolet light with a Leitz Orthoplan microscope. Because this dye stains both apoptotic and non-apoptotic cells we could specifically count the percentage of apoptotic cells displaying chromatin condensation and nuclear fragmentation. For statistical analysis, 100 cells were counted in five different fields. Further characterization of apoptosis was achieved using a commercially available in situ cell death detection kit to find DNA strand breaks using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) reagent according to the manufacturer's protocol (Boehringer Mannheim). The number of TUNEL-positive cells were also counted in five different fields.

![Fig. 2. Hoechst 33258 staining and TUNEL reactivity of cardiac myocytes exposed to 0, 1, 3, and 8 h of hypoxia in the absence of glucose.](image-url)
Analysis of Cytochrome c Release

Subcellular Fractionation—Cardiac myocytes were rinsed with cold PBS, and mitochondrial, and cytosolic (S100) fractions were prepared (45). Briefly, cells were resuspended in 0.25 ml of ice-cold isotonic buffer A (250 mM sucrose, 20 mM HEPES, 1.5 mM MgCl2, 1 mM EDTA, 1 mg/ml phenylmethylsulfonyl fluoride, 8 μg/ml aprotonin, 2 μg/ml leupeptin, 5 μg/ml pepstatin, pH 7.4). The cells were disrupted by two successive sonications on ice for 20 s each with a pause of 1–2 min using a microtip generator set at 40% duty cycle (Sonicator Vibracell, Sonics and Materials, CT). A pellet highly enriched in mitochondria was prepared by centrifugation at 10,000 × g for 30 min. This pellet was resuspended in the same buffer A, and the resulting supernatant was further spun at 160,000 × g for 1 h in a TLA-100 rotor in a Beckman table top ultracentrifuge. The supernatant from this final ultracentrifugation represented the cytosolic fraction.

Western Blot Analysis— Equivalent amounts of mitochondrial and cytosolic fractions were subjected to Western blot analysis as described previously (46). Briefly, the proteins were electrophoresed on 15% SDS-polyacrylamide gels, transferred to Hybond nylon membranes (Amersham Pharmacia Biotech), and immunoblotted with monoclonal antibodies specific for cytochrome c (monoclonal antibody 7H8.2C12 at 1.5 μg/ml; Pharmingen, San Diego). To ensure that cytochrome c release was not caused by a physical disruption of mitochondria, both the mitochondrial and cytosolic fractions were probed with monoclonal antibodies to cytochrome oxidase (subunit IV) (monoclonal antibody 20E8-C12 at a dilution of 0.1 μg/ml; Molecular Probes, Eugene, OR), an enzyme complex bound to the outer leaflet of the inner mitochondrial membrane. Visualization of the signal was by enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech).

Analysis of PARP Cleavage and Immunoblotting

For Western blot analysis of PARP (poly(ADP-ribose) polymerase), both floating and attached cells were rinsed in cold PBS, pH 7.4, and then collected into a defined volume of lysis buffer (62.5 mM Tris, pH 6.8, 8% deionized urea, 10% glycerol, 2% SDS, and protease inhibitors). The cells were then sonicated on ice for 20 s. After the addition of loading buffer the samples were incubated at 65°C for 15 min, and equal amounts of protein were resolved on a 7.5% SDS-polyacrylamide gel. Immunoblotting for PARP was performed (47) using a monoclonal antibody that specifically detects rat, mouse, or human PARP (monoclonal antibody SA-250; clone C-2–10, BIOMOL Research Labs, Inc., Plymouth Meeting, PA) at a 1:5,000 dilution. Visualization of the signal was by ECL.
samples (10 μg) were electrophoresed on 1% agarose gels to visualize laddering.

**Statistical Analysis**

Data are presented as the means ± S.E. The means of numbers of cells undergoing apoptosis were subjected to analysis of variance for multiple comparisons. Paired analysis between two groups was performed by Student’s t test.

**RESULTS**

**Long Term Hypoxia in the Presence of Glucose Induces Apoptosis**—Cardiac myocytes were subjected to hypoxia in the presence of 17.5 mM glucose for 24 h. Genomic DNA was isolated, and 10 μg of DNA was run on a 1% agarose gel. The cardiac myocytes cultured under normoxic conditions in the presence or absence of Oxygenase showed no fragmentation of DNA into oligonucleosomes. However, cardiac myocytes cultured under hypoxic conditions for 24 h showed characteristic internucleosomal DNA fragmentation of approximately 200 base pairs consistent with apoptosis. Preincubation of the cells with 100 μM Z-VAD-fmk, a broad spectrum caspase inhibitor, followed by 24 h of hypoxia, completely prevented DNA laddering (Fig. 1A).

Cultured cardiac myocytes also show strongly positive TUNEL staining. The TUNEL-positive cells showed distinct condensation of the chromatin and fragmented nuclei (Fig. 1B). After 24 h of hypoxia, 16 ± 4% (mean ± S.E.) of cardiac myocytes were apoptotic by these criteria compared with only 4 ± 3% apoptotic cells observed in control, normoxic conditions.

**Short Term Hypoxia in the Absence of Glucose and Other Substrates Also Induces Apoptosis**—To determine if short term hypoxia in the absence of glucose also resulted in induction of apoptosis in the cultured neonatal rat cardiac myocytes, we first examined cell morphology by phase-contrast microscopy and nuclear morphology by Hoechst and TUNEL staining (Fig. 2). Cardiac myocytes cultured in the presence of 17.5 mM glucose were switched to glucose- and pyruvate-free DMEM/F-12 medium and allowed to equilibrate under normoxic conditions for 30 min. Subsequently, these cells were placed in the hypoxia chamber for 1, 3, and 8 h. 3 h of hypoxia resulted in the appearance of a substantial number of apoptotic cells as determined by membrane blebbing, chromatin condensation, and nuclear fragmentation revealed by Hoechst staining (14 ± 3% versus 3 ± 2% apoptotic cells in control, normoxic conditions, n = 5, p < 0.05) (Fig. 3). These cells were also strongly positive by TUNEL (quantitative data not shown). After 8 h of hypoxia, 22 ± 4% cells were apoptotic (n = 5, p < 0.001) (Fig. 3). The cardiac myocytes cultured either in the presence or absence of glucose and pyruvate, under normoxic conditions, showed basal levels of apoptosis of 3 ± 2%.

**Short Term Hypoxia in the Absence of Glucose Clears the Death Substrate PARP**—We next examined whether morphologic features of apoptosis were accompanied by the cleavage of the DNA repair enzyme, PARP, a known substrate for activated caspase 3. Neonatal rat cardiac myocytes cultured in the presence of glucose containing DMEM/F-12 medium were switched to glucose- and pyruvate-free DMEM/F-12 medium and then subjected to hypoxia for 1, 3, or 8 h. Under normoxic conditions only the uncleaved, proform of the enzyme was detected. Hypoxia gradually resulted in the appearance of the characteristic cleaved fragment of approximately 85 kDa, with a dramatic reduction in the amount of proform by 8 h (Fig. 4A).

To test the possibility that PARP cleavage under hypoxic conditions was caspase-dependent, the cardiac myocytes were preincubated with either 100 μM Z-VAD-fmk, a broad spectrum caspase inhibitor, or 100 μM Z-DEVDP-fmk, a more caspase 3-specific inhibitor. As expected, cleavage of PARP was significantly prevented by either caspase inhibitor, with a greater protective effect observed with Z-DEVD-fmk. The nonspecific control peptide Z-FA-fmk had no effect (Fig. 4A).

Genomic DNA isolated from cardiac myocytes subjected to hypoxia for 8 h in the absence of glucose showed distinct labeling confirming the presence of significant apoptosis. Preincubation of cells with the caspase inhibitors Z-VAD-fmk and Z-DEVDP-fmk completely prevented DNA laddering (Fig. 4B).

**Glucose Has a Protective Effect on Hypoxia-induced Apoptosis**—The fact that glucose withdrawal led to significant apoptosis in the cultured cardiac myocytes in response to hypoxia prompted us to examine the role this substrate was playing in the apoptotic process. Readdition of glucose to the medium at a final concentration of 17.5 mM and then preincubation of cells under normoxic conditions for 30 min followed by hypoxia of 1, 3, and 8 h prevented apoptosis as demonstrated by the lack of PARP cleavage (Fig. 5), DNA laddering (Fig. 4B), as well as cell morphology (data not shown). To elucidate further the precise concentration at which glucose achieved its protective effect, a dose-response experiment was performed. Glucose at a concentration of −1.0 mM prevented PARP cleavage with a maximal effect reached at 7.5 mM glucose (Fig. 6).

**Pyruvate, Lactate, and Propionate Do Not Prevent Hypoxia-induced Apoptosis**—We next tested the possibility that other
intermediary sources of energy metabolism protected cells from hypoxia-induced apoptosis. Cardiac myocytes were switched to glucose- and pyruvate-free medium. Pyruvate, lactate, and propionate were added to the medium, and the cells were preincubated for 30 min under normoxic conditions. Then the cells were subjected to hypoxia for 3 h. As shown in Fig. 7, none of the three substrates prevented PARP cleavage. Similarly, none of these alternate substrates prevented morphologic changes of apoptosis (not shown). A dose-response curve of pyruvate showed that concentrations up to 10 mM failed to prevent PARP cleavage and hypoxia-mediated apoptosis (Fig. 7).

Hypoxia in the Absence of Glucose Is Accompanied by Translocation of Cytochrome c to Cytosol—Hypoxia for 3 h in the absence of glucose led to substantial cytochrome c release from the mitochondria into the cytosol of cultured cardiac myocytes as revealed by Western blot analysis of the cytosolic fraction (Fig. 8A), with a more dramatic release observed at 8 h of hypoxia as documented by a decrease in cytochrome c signal in the mitochondrial fraction (Fig. 8B). Consistent with the data obtained with PARP cleavage and cell nuclear morphology, glucose prevented cytochrome c release. Further Western blot analysis showed that cytochrome oxidase was present only in the mitochondrial fraction, never in the cytosolic fraction, indicating that mitochondria remained intact and that no cross-contamination of cellular fractions occurred experimentally.

Preincubation of the cultured cardiac myocytes with the caspase inhibitors Z-VAD-fmk and Z-DEVD-fmk for 30 min followed by hypoxia in the absence of glucose showed that Z-VAD-fmk did not prevent cytochrome c release, but Z-DEVD-fmk did consistently block cytochrome c release in response to hypoxia (Fig. 8A), suggesting that caspase 3, or a similar protease, participated in stimulating cytochrome c release.

Glycolytic Inhibitors 2-Deoxyglucose and Iodoacetate Reverse the Glucose-induced Prevention of Hypoxia-induced Apoptosis—Cardiac myocytes were incubated in glucose-containing medium plus either 3 mM 2-deoxyglucose or 4 mM iodoacetate, both of which are glycolytic inhibitors. The former accumulates as 2-deoxyglucose-6-PO₄, which cannot be metabolized by hexokinases, and the latter blocks glycolysis by inhibiting the enzyme glyceraldehyde-3-phosphate-dehydrogenase in the glycolytic pathway. Both 2-deoxyglucose and iodoacetate reversed the glucose-induced prevention of hypoxia-induced apoptosis as documented by PARP cleavage, morphologic changes, cytochrome c release, and DNA laddering (Fig. 9).

Cardiac Fibroblasts Do Not Undergo Apoptosis in Response to Hypoxia—To address the possibility that the small (2–3%) contamination of fibroblasts in our Percoll-enriched myocyte-rich cultures potentially contributed to the observed effects of apoptosis in cultures of cardiac myocytes subjected to hypoxia, highly enriched cultures of cardiac non-myocytes (mostly fibroblasts) were prepared by two passages of cells obtained from the upper Percoll density gradient. These cells were subsequently plated on 100-mm dishes and then subjected to hypoxia for 0–24 h. Cardiac fibroblasts either in the presence or absence of glucose showed no DNA laddering (Fig. 10) or morphologic changes of apoptosis (not shown) in response to hypoxia.

**DISCUSSION**

In the present study we have shown that hypoxia-induced apoptosis in neonatal rat cardiac myocytes is accompanied by...
FIG. 9. Apoptosis of cardiac myocytes in the presence of 17.5 mM glucose and glycolytic inhibitors (2-deoxyglucose (2-DG) or iodoacetate (Io)) after 8 h of hypoxia. 2-Deoxyglucose and iodoacetate were used at a final concentration of 3 mM and 4 mM, respectively. Panel A, TUNEL reactivity (representative of three independent experiments). Panel B, Western blot analysis of cytochrome c release (representative of two experiments). Panel C, Western blot analysis of PARP cleavage (representative of two independent experiments). Panel D, genomic DNA fragmentation was present in cardiac myocytes exposed to 8 h of hypoxia in the presence of glucose and glycolytic inhibitors, 2-deoxyglucose or iodoacetate.
activation of the terminal apoptotic machinery comprised of cytochrome c release, effector caspase activation, and cleavage of the death substrate PARP. We have also demonstrated that the presence of extracellular glucose, but not other substrates, protects cardiac myocytes from hypoxia-induced apoptosis, prevents cytochrome c release and caspase activation, and that this protection appears to depend on glycolytic metabolism. Although previous reports have documented apoptosis in cardiac myocytes subjected to hypoxia or ischemia (9, 29), this is the first report to demonstrate that hypoxia causes cytochrome c release and that glucose can prevent hypoxia-induced apoptosis.

Apoptosis clearly occurs in myocardium during and after ischemia (49–51), although the relative contribution of apoptotic and non-apoptotic cell death to ischemic cardiac injury is still unclear (49, 52). Many recent studies have indicated that either ischemia or hypoxia alone (26, 53) or in combination with reoxygenation (10, 51, 54) can trigger cardiomyocyte apoptosis, especially in ischemic border regions (49). The mechanism by which hypoxia induces apoptosis remains unclear but is probably analogous to the pathways by which other cellular stresses initiate apoptosis. Cellular stresses, such as growth factor withdrawal, UV irradiation, or treatment with actinomycin D, glucocorticoids, or chemotherapeutic agents, cause apoptosis via a mitochondria-dependent release of cytochrome c and subsequent activation of the Apaf-1-caspase 9 complex or apoptosisome (55). Caspase 9 subsequently cleaves and activates downstream effector caspases such as caspase 3, which then mediate the biochemical features of apoptotic cell death (e.g., PARP and lamin cleavage). The triggers for cytochrome c release may include reactive oxidant species, increased cytoplasmic calcium concentration, decreased ATP levels, as well as activation or increased expression of the proapoptotic proteins p53 and Bax (55).

Hypoxia and ischemia appear to induce a similar set of changes. Hypoxia has been shown to induce hypoxia-inducible factor-1α, which in turn increases p53 levels (56). It has been demonstrated that p53 also stimulates cytochrome c release through its action on the pro-apoptotic protein Bax (57, 58). Our findings show that hypoxia causes cytochrome c release from mitochondria in neonatal cardiac myocytes, which results in downstream caspase activation, PARP cleavage, and DNA laddering. In our system, reoxygenation was not required to induce these changes, thereby suggesting that reactive oxidant species were not critical in triggering apoptosis. We have not yet tested whether hypoxia-inducible factor-1α is responsible for induction of p53 and cytochrome c release in our system.

It has been shown recently that apoptosis can result in cardiac myocyte apoptosis in a p53-independent manner, as well (50). This suggests that another pathway may also be triggered by hypoxia. In many cells, apoptosis can be induced by a death receptor-mediated pathway that activates effector caspases by recruitment and autoactivation of caspase 8 and does not entail cytochrome c release (59). A number of reports have implicated a similar receptor-mediated apoptosis in hypoxic and ischemic cardiac myocytes. For example, ischemia induces increased expression of Fas (CD95) (9, 28). However, there is no evidence for increased Fas ligand levels in ischemic cardiac regions or for Fas ligand expression and release by hypoxic cardiomyocytes. Thus, this alternate pathway may be less important, at least in the cultured cardiomyocyte system.

A number of recent reports have shown that the release of cytochrome c from the mitochondria to the cytosol initiates caspase 3 activation through the aggregation of Apaf-1, procaspase 9, and dATP and subsequent activation of caspase 9, which in turn activates caspase 3 (25, 55). In the present study, hypoxia of cardiac myocytes in the absence of glucose caused release of cytochrome c, with the resultant appearance of apoptosis characterized by PARP cleavage and DNA fragmentation. An intriguing observation in the present study was that Z-DEVD-fmk, a caspase 3-specific inhibitor, partially blocked cytochrome c release. This finding suggests that activated caspase 3 results in additional release of cytochrome c, which may amplify the death stimulus (55). Caspase inhibitors do not prevent cytochrome c release induced by several other extracellular stressors, including UV irradiation and apoptogenic agents such as staurosporine (55) or from overexpression of Bax (60). This feedback amplification of cytochrome c release by caspase 3 appears to take place by caspase 3-mediated cleavage of Bcl-2 (61), which normally acts to prevent cytochrome c release (62).

Glucose uptake and metabolism have long been shown to be protective for ischemic myocardium (38, 39). Ischemic and hypoxic cardiomyocytes increase the sarcolemmal expression of facilitative glucose transporters GLUT-4 (40) and GLUT-1 (41, 63) and increase the activity of hexokinase II (64). These changes would serve to enhance glucose uptake and metabolism by ischemic and hypoxic cardiomyocytes. We have shown recently that hypoxic vascular smooth muscle cells in which GLUT-1 was overexpressed were protected from hypoxia-induced apoptosis.2 GLUT-1 overexpression also substantially reduced signaling through the stress-activated protein kinase pathway, and decreased mitogen-activated protein kinase kinase-1 and c-Jun NH2-terminal kinase activities appeared to account for some of the reduction in apoptosis. In addition, the GLUT-1-overexpressing cells showed an improved ability to sequester intracellular calcium, which may have contributed to the prevention of apoptosis in these cells.2 Now, in this report,

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2 Z. Lin, J. M. Weinberg, R. Malhotra, S. E. Merritt, L. B. Holzman, and F. C. Brosius III, submitted for publication.
Glycolysis and Hypoxia-induced Apoptosis in Cardiomyocytes

we show that glucose uptake and metabolism protected cardiomyocytes from hypoxia-induced apoptosis. Moreover, it appears that glycolysis and not some other metabolic pathway accounted for the protective response and that other metabolic substrates (pyruvate, lactate, propionate) provided no protection from apoptosis.

In summary, hypoxia in the absence of glucose of cultured neonatal rat cardiac myocytes is accompanied by significant release, but other mechanisms such as direct effects on hypoxia-inducible factor-1α or p53 activities or on antiapoptotic pathways, such as those mediated by phosphatidylinositol 3-kinase and the serine/threonine kinase c-Akt/protein kinase B, will need to be examined.

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