Cardioprotective effect of chronic hyperglycemia: effect on hypoxia-induced apoptosis and necrosis

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The long-term mortality rate for diabetic patients after a myocardial infarction is significantly elevated relative to that of the nondiabetic patient (1). Although recurring myocardial infarction and enhanced susceptibility to arrhythmias are potential candidates for the observed clinical results, epidemiologists have concluded that the dominant cause for the elevated mortality rate is the development of congestive heart failure (1). Because larger infarcts increase the risk of developing congestive heart failure, numerous studies have focused on the influences of diabetes on ischemic injury (8, 30). These studies have yielded mixed results, an outcome not altogether surprising considering the wide array of experimental conditions employed. Not only has the severity of the diabetic condition been varied, but the end point measured, the composition of the perfusion medium used, and the nature of the ischemic insult employed have been varied as well. Despite apparent inconsistencies in the experimental results, the studies have established that mild diabetes favorably influences the outcome of an ischemic event (8, 20, 30). Moreover, the outcome of an ischemic event depends on a combination of competing factors that are modulated by diabetes (8, 30).

One of the factors affecting the sensitivity of the diabetic heart to an ischemic insult is glucose. Indeed, glucose-insulin-potassium therapy was once considered a promising regimen for the treatment of ischemia (36). It was believed that glucose would provide a source of ATP production under conditions favoring ATP hydrolysis. However, it was subsequently discovered that glucose metabolism also increased the production of H+ during ischemia, causing an elevation in intracellular Ca2+ concentration (27). Moreover, acute hyperglycemia abolished the beneficial effects of ischemic preconditioning (16). Thus many investigators now believe that the acute effects of glucose increase the sensitivity of the heart to an ischemic event.

However, glucose also possesses important chronic actions. First, it modulates Ca2+ movement, an effect that could affect the ischemic heart by altering the degree of Ca2+ overload (15, 17, 33). Second, chronic hyperglycemia activates protein kinase C in several cell types (19, 40). Although the activity of protein kinase C in the glucose-treated cardiomyocyte has not been examined, it is relevant that protein kinase C activity is increased in the diabetic heart (10). Moreover, the activation of protein kinase C in many cells shifts the cellular machinery in favor of survival and proliferation (11). Indeed, the activation of protein kinase C has been touted as a major factor contributing to ischemic preconditioning (38). Therefore, the chronic actions of glucose, unlike some of its acute actions, should decrease the susceptibility of the myocyte to an ischemic insult.

Ischemic cell death in the heart is triggered by two processes, apoptosis and necrosis (3, 13). These two principal modes of cell death differ with respect to mechanism, sequelae, and type of damage (21, 22). Necrosis is characterized by cell swelling, organelle damage, ATP depletion, Ca2+ accumulation, random phosphorylation; calcium overload
DNA fragmentation, and rupture of the cell membrane. Because intracellular constituents are released from the necrotic cell, an acute inflammatory response is initiated. By contrast, apoptosis is associated with cell shrinkage, DNA fragmentation at linker regions, cell budding, and the formation of apoptotic bodies, which are phagocytosed by macrophages. Because intracellular constituents are not released during apoptosis, the apoptotic cell is involuted without triggering an acute inflammatory response.

Kajstura et al. (14) argued that apoptosis is the dominant mechanism of cell death in rats undergoing coronary artery occlusion. Apoptosis may also make a major contribution to overall cell death in the infarcted human heart (29) and is an important pathological feature of the hypoxic myocyte (37) and the reperfused myocardium (9, 12). Although the potential role of apoptosis in ischemic injury has only recently been investigated, previous studies have clearly shown that necrosis plays a central role in ischemia-induced myocardial damage. Therefore, the aim of the present study was to examine the effect of chronic glucose exposure on hypoxia-induced necrosis and apoptosis.

METHODS

Cardiomyocyte preparation and incubation conditions. Neonatal cardiomyocytes were prepared from 2-day-old Wistar rats by the procedure of McDermott and Morgan (25), as described previously (33). The dissociated cells were preplated on plastic culture flasks for 90 min at 37°C to allow the attachment of nonmyocytes. The cells were then resuspended in MEM supplemented with vitamins, antibiotics, amino acid solutions, 10% newborn calf serum, and 0.1 mM 5-bromo-2-deoxyuridine (25). The cells were plated at a density of $10^5$ cells/dish (10 cm diameter) onto glass chamber slides or dishes precoated with 0.1% gelatin. After an overnight incubation to allow the attachment of viable cardiomyocytes, the cells were cultured in serum-free medium containing MEM supplemented with 5 mM (control) or 25 mM glucose (glucose treated). The concentration of glucose chosen for the glucose-treated cells corresponded to the concentration found in the plasma of type I diabetic rats and has been used in previous studies examining the influence of hyperglycemia on the isolated cardiomyocyte (7, 31). All experiments began after 3 days in control culture. The cells beat spontaneously, although in some experiments, butanedione monoxime was added to inhibit the spontaneous contractions. Generally, the cells were not confluent, which allowed the examination of individual cells.

To induce the hypoxic insult, the cells were transferred to medium lacking glucose but containing 10 mM deoxyglucose and 3 mM amobarbital (Amytal). The cells were placed in a cell culture incubator equilibrated with 2.3% O2-5% CO2 and 3 mM amobarbital. The cells were then resuspended in MEM supplemented with 5 mM (control) or 25 mM glucose (glucose treated). The concentration of glucose chosen for the glucose-treated cells corresponded to the concentration found in the plasma of type I diabetic rats and has been used in previous studies examining the influence of hyperglycemia on the isolated cardiomyocyte (7, 31). All experiments began after 3 days in control culture. The cells beat spontaneously, although in some experiments, butanedione monoxime was added to inhibit the spontaneous contractions. Generally, the cells were not confluent, which allowed the examination of individual cells.

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Western blot analysis. The cellular content of nonphosphorylated and phosphorylated B–d–2, Bax, and Bad was determined by Western blot analysis. After treatment with trypsin to remove the cells from the dishes, the cells were suspended in HEPES buffer (10 mM) containing 2 mM EDTA, 0.1 mg/ml phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate. They were immediately lysed by sonication. The cell lysate was then centrifuged at 10,000 g, and the supernatant was utilized for Western blot analysis. When phosphorylated Bad, phosphorylated B–d–2, or phosphorylated Bax content was determined, the lysates were first immunoprecipitated with antibody directed against the appropriate B–d–2 family member. After solubilization, the samples were subjected to SDS-polyacrylamide gel (13%) electrophoresis. Each lane was loaded with 10 μg of protein. When total B–d–2, Bax, and Bad content was measured, the initial immunoprecipitation step was eliminated, and the lysates were initially subjected to the electrophoresis step. All samples were then transferred to nitrocellulose membranes, where they were blocked. The membranes were then incubated with the appropriate antibody, which was anti-phosphoserine when phosphorylated Bad was measured but was anti-Bad when total Bad content was evaluated. After a washing step, the membranes were incubated with a secondary antibody, goat anti-rabbit IgG, and again washed. The Western blot was detected by the enhanced chemiluminescence reaction. The blots were analyzed by a computer program, which detects the area under the curve.

Statistical analysis. The statistical significance of the data was determined using the Student’s t-test for comparison within groups and ANOVA combined with Tukey’s post hoc test for comparison between groups. P < 0.05 was considered statistically significant.

RESULTS

The effect of chronic hyperglycemia on hypoxic injury was examined using isolated myocytes incubated for 3 days with medium containing 5 mM (control) or 25 mM glucose (glucose treated). To induce hypoxia, the cells were transferred to medium lacking glucose but containing 10 mM deoxyglucose and 3 mM amobarbital. Deoxyglucose was added to prevent glucose metabolism from influencing the outcome of the hypoxic insult. After 1 h of chemical hypoxia, the degree of cell necrosis was determined by the trypan blue exclusion method and by monitoring the percentage of cells that underwent shrinkage. After 1 h of chemical hypoxia, 28% of the control cells were unable to exclude trypan blue (Fig. 1). An equal number of the control cells (26%) swelled an average of 13% during the hypoxic insult. Because cell swelling and trypan blue exclusion are measures of cell necrosis (6), the excellent agreement between the two procedures indicates that ~27% of the control cells undergo necrosis during the first hour of hypoxia.

The hypoxic insult also dramatically increased the number of cells that exhibited DNA fragmentation, as evidenced by positive TdT-mediated dUTP nick end labeling (TUNEL; Fig. 2). Although only 8% of the control cells showed a positive brown TUNEL pattern before hypoxia, the number of positively stained cells increased to 33% after 1 h of hypoxia (Fig. 2). TUNEL has been commonly used to monitor the extent of cellular apoptosis, although its specificity has been recently questioned (28). Therefore, two other procedures, DNA laddering and cell shrinkage, were utilized to monitor the extent of cellular apoptosis. After 1 h of chemical hypoxia, 32% of the control cells lost cell volume (Fig. 2), with the average decline in cell volume being 17%. The control, hypoxic cells also exhibited
prominent DNA laddering (Fig. 3), supporting the idea that chemical hypoxia triggered apoptosis in nearly one-third of the hypoxic cells.

Significant protection against hypoxia-induced apoptosis and necrosis was observed in cells pretreated with high glucose before the hypoxic insult. As seen in Fig. 1, only 10% of the glucose-treated cells accumulated trypan blue after 1 h of hypoxia. Similarly, 13% of the glucose-treated cells swelled during the hypoxic insult, with the average increase in cell volume being 12%. Because glucose treatment affected the number of cells that swelled without altering the extent of swelling (Fig. 1), it is logical to conclude that hyperglycemia blocks an early step in the necrotic pathway.

Glucose pretreatment also reduced from 33% to 12% the number of hypoxic cells that shrunk and exhibited positive TUNEL staining (Figs. 2). Because the two properties are characteristic features of apoptotic cells, glucose treatment appears to reduce the number of cells undergoing apoptosis by two-thirds. Consistent with this conclusion was the observation that the intensity of the DNA ladder was diminished in the hypoxic, glucose-treated cells in comparison to the hypoxic, control cells (Fig. 3). Nonetheless, the extent of cell shrinkage was unaffected by glucose treatment (16 and 17% shrinkage in glucose-treated and control cells, respectively), implying that the ant apoptotic activity of hyperglycemia involves a step that precedes cell shrinkage.

One of the most widely discussed mechanisms for the initiation of hypoxia-induced cell death is Ca\(^{2+}\) overload (35). To determine whether glucose treatment provided protection by attenuating the degree of hypoxia-induced Ca\(^{2+}\) overload, glucose-treated and control cells were first loaded with the Ca\(^{2+}\) indicator indo 1, and then baseline indo 1 fluorescence at 410 and 490 nm was measured. Before the hypoxic insult, no difference in F\(_{410}/F_{490}\) was observed between the glucose-treated and control cells (Fig. 4). However, after 1 h of hypoxia, F\(_{410}/F_{490}\) increased 72% in the control cells but was not significantly changed in the glucose-treated cell (Fig. 4). Therefore, after 1 h of hypoxia, the glucose-treated cell contained ~40% less Ca\(^{2+}\) than the control cell.

Although cellular accumulation of Ca\(^{2+}\) during ischemia or hypoxia has often been attributed to a stimulation in Ca\(^{2+}\) influx via the concerted actions of the Na\(^+\)/H\(^+\) and Na\(^+\)/Ca\(^{2+}\) exchangers (15), the cell contains other potential regulators of Ca\(^{2+}\) movement in the cell, including the proteins belonging to the Bcl-2 family (2,4). These proteins are also of interest, because they serve as primary regulators of apoptosis. As seen in Fig. 5, glucose treatment dramatically elevated the levels of the cytoprotective protein Bcl-2. On the other hand, Western blot analysis revealed that glucose treatment had no significant effect on cellular Bax, a Bcl-2 family protein capable of reversing the effects of Bcl-2 (Fig. 5).

Although an increase in the Bcl-2-to-Bax ratio is considered cytoprotective, the degree of protection depends on the activity of the regulatory proteins. One of the major modes of regulating the cytoprotective and cytotoxic activity of these proteins is through changes in their phosphorylation status (4). Therefore, the effect of glucose treatment on the phosphorylation status of Bcl-2, Bax, and a related protein, Bad, was examined.

![Image](http://ajpheart.physiology.org.org/)

**Fig. 4.** Effect of glucose treatment on hypoxia-induced elevations in intracellular Ca\(^{2+}\) concentration. Glucose-treated and control neonatal cardiomyocytes were loaded with Ca\(^{2+}\) indicator dye indo 1. Under normoxic conditions, fluorescence at 410 and 490 nm of specific cells was measured using point scan mode of confocal microscope. Fluorescence of these same cells was monitored after a 1-h hypoxic insult. Data are expressed as fluorescence ratio (F\(_{410}/F_{490}\)), which is a measure of intracellular Ca\(^{2+}\) concentration. Values are means ± SE of 3–4 preparations, with 10 cells examined per preparation. *Significant difference between control and glucose-treated cells after hypoxic insult (P < 0.05). Hypoxia led to a significant increase in cellular Ca\(^{2+}\) content of control, but not glucose-treated, cell.
Although glucose treatment had a minimal effect on the phosphorylation status of Bcl-2 and Bax (data not shown), a significant increase in the phosphorylation state of the proapoptotic factor Bad was observed (Fig. 6A). The elevation in the phosphorylation state of Bad was not related to an increase in total Bad content (Fig. 6, B and C), suggesting that a significant shift toward the inactive, phosphorylated form of Bad occurs in the glucose-treated cell.

**DISCUSSION**

The present study showed that chronic exposure of the cardiomyocyte to hyperglycemia rendered the cell resistant to hypoxia-induced necrosis and apoptosis (Figs. 1–3). One of the putative beneficial mechanisms of hyperglycemia is a reduction in flux through the Na+/H+ and Na+/Ca2+ exchangers (8). According to the scheme outlined by Karmazyn and Moffat (15), H+ are generated from lactate production and ATP breakdown in the ischemic heart. Excessive H+ production causes the cellular influx and accumulation of Na+ through the actions of the Na+/H+ exchanger. Although intracellular Na+ accumulation has no particular toxic effect outside some adverse osmotic effects, Karmazyn and Moffat argued that Na+ overload is indirectly cytotoxic, because it leads to Ca2+ overload.

The significance of the exchanger-induced Ca2+ overload mechanism is dramatically diminished in the diabetic heart because of reductions in the activities of the Na+/H+ and Na+/Ca2+ exchangers and the resulting attenuation of ischemia-induced Ca2+ overload (8). Indeed, Khandoudi et al. (17) found that the resistance of the mildly diabetic cell to an ischemic insult is largely attributed to the reduction in proton-linked Ca2+ accumulation. Several lines of evidence suggest that the glucose-treated myocyte becomes resistant to a hypoxic insult through a similar mechanism. First, the...
glucose-treated cell accumulates less Ca\(^{2+}\) during chemical hypoxia than the control cell (Fig. 4). Second, although the activity of the Na\(^+\)/H\(^+\) exchanger has not been examined in the glucose-treated myocyte, chronic hyperglycemia is known to suppress Na\(^+\)/H\(^+\) exchanger activity in some cell types (39). Moreover, exposure of the isolated cardiomyocyte to chronic hyperglycemia induces a diabetes-like phenotype and would, therefore, be expected to reduce Na\(^+\)/H\(^+\) exchanger activity in some cell types (7, 31). Third, the activity of the Na\(^+\)/Ca\(^{2+}\) exchanger is reduced in the isolated cardiomyocyte after glucose treatment (33).

Ca\(^{2+}\) overload initiates several toxic reactions, including mitochondrial disruption, activation of Ca\(^{2+}\)-dependent hydrolases and ATPases, and contracture-induced structural damage. Although the present study did not focus on the mechanism behind Ca\(^{2+}\)-induced cellular death, it is interesting that glucose treatment protects against necrosis and apoptosis. Two Ca\(^{2+}\)-like mechanisms may contribute to both death processes, namely, the disruption of mitochondrial function and the activation of Ca\(^{2+}\)-dependent enzymes (18, 24, 35). Mitochondrial dysfunction leading to the release of proapoptotic factors represents an early event in the apoptotic pathway, whereas the activation of the hydrolases occurs in the irreversible third phase of apoptosis. Ca\(^{2+}\) excess is also involved in an early phase of necrotic cell death (35). Therefore, it is logical to assume that the prevention of Ca\(^{2+}\) overload by glucose treatment contributes to the reduction in hypoxia-induced necrosis and apoptosis. In support of this notion, Chen et al. (5) showed that Ca\(^{2+}\) channel blockers reduced the degree of myocyte apoptosis induced by chemical hypoxia.

Another cardioprotective mechanism of glucose treatment is the upregulation of Bcl-2, which is capable of interfering with hypoxia-induced cell death through at least two mechanisms. First, the modulation of Ca\(^{2+}\) movement by Bcl-2 is thought to be cytoprotective. According to Baffy et al. (2), the upregulation of Bcl-2 in some cells prevents excessive accumulation of Ca\(^{2+}\) by the mitochondria, an effect favoring cell survival. Similarly, Murphy et al. (26) showed that Bcl-2 inhibits Ca\(^{2+}\)-induced respiratory damage that occurs during mitochondrial Ca\(^{2+}\) overload. A second major cytoprotective action of Bcl-2 is the stabilization of the mitochondria, preventing the release of proapoptotic factors (4, 13). Finally, Bcl-2 reduces the degree of oxidative stress (13). The net effect of these three actions is an attenuation in mitochondrial disruption and protection against necrosis and apoptosis. Indeed, Shimizu et al. (34) showed that KCN-induced necrosis is blocked by over-expression of Bcl-2. Moreover, Bcl-2 overexpression protects neurons against an ischemic insult (23).

Although Bcl-2 is a cytoprotective and antiapoptotic factor, several of the Bcl-2 family members, such as Bax and Bad, are proapoptotic. The balance between the proapoptotic and antiapoptotic factors determines whether cells proliferate or die. In the glucose-treated cell, Bcl-2 levels are dramatically elevated, whereas the levels of the proapoptotic factors, Bax and Bad, remain unaltered, a condition that tends to shift the balance within the cell in favor of survival (4). However, the activity of these Bcl-2 family members is regulated by protein phosphorylation. This type of regulation has been most widely studied in neurons, where the phosphorylation of the proapoptotic factor Bad can be an important process shifting the cell’s balance in favor of survival (4). Thus the evidence that glucose treatment enhances the phosphorylation of Bad represents another likely mechanism underlying the cardioprotective activity of chronic hyperglycemia. Although the identity of the protein kinase responsible for maintaining an elevated phosphorylation state of Bad has not been established, it is interesting that one of the prominent protein kinases that is upregulated by diabetes and hyperglycemia is protein kinase C (10, 19, 40). Because protein kinase C activation is generally considered cardioprotective, it would be attractive to suggest that the cardioprotective effects of protein kinase C are related in part to the inactivation of Bad.

Although the present and previous studies clearly support the notion that chronic hyperglycemia and diabetes can provide protection against ischemia and hypoxia-induced cell death (8), it does not address the status of the surviving cells. This is an important issue, because the mortality rate of the diabetic patient after a myocardial infarction is greater than that of the nondiabetic patient (1). This elevated mortality rate has been largely attributed to the development of a cardiomyopathy. The present study supports the notion that the increased risk of developing congestive heart failure is linked to the preexisting diabetic cardiomyopathy (1). This scenario would imply that chronic hyperglycemia and diabetes act as a two-edged sword: on the one hand, they render the cell resistant to an ischemic or hypoxic insult, and on the other hand, the surviving cell exhibits abnormal contractile and transport properties, which increase its risk of eventually developing severe pump failure.

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