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

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Schaffer, Stephen W., Cherry Ballard Croft, and Viktoria Solodushko. Cardioprotective effect of chronic hyperglycemia: effect on hypoxia-induced apoptosis and necrosis. Am J Physiol Heart Circ Physiol 278: H1948–H1954, 2000.—It is generally accepted that mild forms of diabetes render the heart resistant to an ischemic insult. Because myocytes incubated chronically in medium containing high concentrations of glucose (25 mM) develop into a diabetes-like phenotype, we tested the hypothesis that high-glucose treatment diminishes hypoxia-induced injury. In support of this hypothesis, we found that cardiomyocytes incubated for 3 days with medium containing 25 mM glucose showed less hypoxia-induced apoptosis and necrosis than cells exposed to medium containing 5 mM glucose (control). Indeed, whereas 27% of control cells became necrotic after 1 h of chemical hypoxia with 10 mM deoxyglucose and 5 mM amobarbital (Amytal), only 11% of the glucose-treated cells became necrotic. Similarly, glucose treatment reduced the extent of apoptosis from 32% to 12%. This beneficial effect of glucose treatment was associated with a 40% reduction in the Ca2+ content of the hypoxic cell. Glucose treatment also mediated an upregulation of the cardioprotective factor Bcl-2 but did not affect the cellular content of the proapoptotic factors Bax and Bad. Nonetheless, the phosphorylation state of Bad was shifted in favor of its inactive, phosphorylated form after high-glucose treatment. These data suggest that glucose treatment renders the cardiomyocyte resistant to hypoxia-induced apoptosis and necrosis by preventing the accumulation of Ca2+ during hypoxia, promoting the upregulation of Bcl-2, and enhancing the inactivation of Bad.

apoptosis; diabetes; Bcl-2 family; cardiomyocyte; Bad phosphorylation; calcium overload

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
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 × 10^6 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 media containing MEM lacking glucose but containing 10 mM deoxyglucose (normal) nuclei. 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 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% O_2-5% CO_2-balance N_2.

Calcein fluorescence analysis. Before the hypoxic insult, some cells were incubated with medium containing 5 µM calcein-AM for 30 min. The calcein-loaded cells were then washed three times with calcein-free medium and placed in standard serum-free media containing 5 or 25 mM glucose. Each cell served as its own control. The volume of a series of cells was determined before and after 1 h of hypoxia. To measure cell volume, a confocal microscope was used to scan calcein fluorescence at various depths within the cell. From the surface area of the scan and the pixel thickness of each confocal slice, the cell volume was calculated (32). The average cell volume of the glucose-treated and control cells before the hypoxic insult was 5.4 ± 0.2 × 10^3 and 6.4 ± 0.2 × 10^3 µm³, respectively. Because specific cells were monitored individually by the confocal microscope, secondary necrosis of cell fragments could be distinguished from primary necrosis, the latter seen as a net increase in cell volume. Secondary necrosis was not included in the calculation of the percent cell necrosis. The confocal microscope procedure was accurate within 2%; therefore, a deviation of >5% from the prehypoxic value was required to classify a cell as shrunken (necrotic) or swollen (apoptotic).

Cellular Ca²⁺ content. Cardiomyocytes were loaded with 2 µM indo 1 for 20 min at 37°C. The loading and subsequent rinsing steps took place before the onset of the hypoxic insult. To prevent oscillations in Ca²⁺ content due to cell beating, the cells were treated with 20 mM 2,3-butanedione monoxime, an agent effective in blocking the interaction of the muscle proteins. Indo 1 fluorescence at 410 and 490 nm was measured using the point scan mode of the confocal microscope. Data obtained before the hypoxic insult and after 1 h of hypoxia without reperfusion were expressed as the fluorescence ratio (F_410/F_490), a measure of intracellular Ca²⁺ concentration. Before the hypoxic insult, indo 1 fluorescence of the glucose-treated and control cells was not significantly different. Each cell served as its own control.

Trypan blue staining and Tdt-mediated dUTP nick end labeling procedure. To measure cell viability, hypoxic and normoxic cells were incubated for 5 min at room temperature with medium containing 0.4% trypan blue. After 5 min of incubation, the cells were fixed in Krebs-Henseleit buffer containing 2% glutaraldehyde. Four separate fields in the light microscope were used to assess trypan blue staining.

The Klenow Frag EL DNA fragmentation detection kit was used to monitor end-labeled DNA fragments of apoptotic nuclei. After the 3-day standard incubation and 1 h of hypoxia, some glass slides were fixed in 4% formaldehyde for 15 min and then resuspended in 80% ethanol for 20 min. After rehydration in PBS, the samples were permeabilized by incubation for 5 min with 20 µg/ml proteinase K in 10 mM Tris (pH 8.0). The slides were then rinsed in PBS. Endogenous peroxidases were inactivated by exposing the samples to 3% H₂O₂ for 5 min. After the cells were rinsed, the slides were placed in 1 × Klenow buffer for 20 min. The Klenow labeling reaction mixture was then added to the samples and allowed to incubate at 37°C for 1.5 h. The reaction was terminated by a 5-min incubation with buffer containing 0.5 mM EDTA (pH 8.0) followed by a 10-min exposure to blocking buffer. The samples were then exposed for 30 min to a peroxidase-streptavidin conjugate. After a 15-min incubation with 0.7 mg of 3,3′-diaminobenzidine and 0.6 mg of H₂O₂-urea, the samples were rinsed and then counterstained with hematoxylin. For color fixation, the glass slides were briefly immersed in 100% ethanol and then in xylene. The samples were analyzed by light microscopy, with four separate fields used in counting the dark brown (apoptotic) and purple (normal) nuclei.

DNA ladder analysis. DNA was isolated from normoxic and hypoxic cells that had been scraped from their incubation dishes. DNA was isolated according to the method described in the DNA apoptosis ladder kit (Boehringer-Mannheim). The concentration of isolated DNA was determined by measuring the absorbance at 260 nm. The isolated DNA (10 µg) was subjected to agarose gel (2%) electrophoresis. The gel was stained with 2 µg/ml ethidium bromide. After the gel was destained for 20 min, the DNA was visualized under ultraviolet light.
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...
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 \(\sim 40\%\) 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.
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. 6B 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

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**Fig. 5. Effect of glucose treatment on cellular levels of Bcl-2 and Bax.**

Glucose-treated and control cells were lysed, and lysate was subjected to SDS-PAGE. After transfer to nitrocellulose membranes, samples were incubated with antibodies directed against Bcl-2 or Bax. A: Western blots detected by enhanced chemiluminescence reaction after exposure to goat anti-rabbit IgG. Lane 1, Bax and Bcl-2 in control cells; lane 2, glucose-treated cells. Blots were analyzed by a computer, which measured area under curve. B: relative levels of Bax and Bcl-2. Value of 1 was assigned to bands corresponding to control Bcl-2 and control Bax. Values are means ± SE of 9 different preparations.

**Fig. 6. Effect of glucose treatment on phosphorylation status of Bad.**

Glucose-treated and control cells were lysed, and lysate was immunoprecipitated with antibody directed against Bad. Solubilized samples were subjected to SDS-PAGE. After transfer to nitrocellulose membranes, samples were incubated with an antibody directed against phosphoserine residues. Western blots for phosphorylated Bad were analyzed as described in Fig. 5. Content of phosphorylated Bad was determined using procedure described in Fig. 5, except antibody was directed against Bad. A and B: representative Western blots for phosphorylated Bad and total Bad, respectively, reveal an elevation in phosphorylated Bad in glucose-treated cells (lane 2) vs. control cells (lane 1). C: relative levels of phosphorylated Bad and total Bad. Value of 1 was assigned to band corresponding to control Bad and control phosphorylated Bad. Values are means ± SE of 6 different preparations. Content of phosphorylated Bad was elevated in glucose-treated cell; total Bad content was unaffected by glucose treatment.
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 (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 overexpression 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 non diabetic 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.

This study was supported by a grant from the American Heart Association.

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Received 13 September 1999; accepted in final form 20 December 1999.

REFERENCES


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