Hyperglycemia exaggerates ischemia-reperfusion–induced cardiomyocyte injury: Reversal with endothelin antagonism

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Objectives: We have previously demonstrated an importance of endothelin-1 in diabetic patients undergoing bypass surgery. Recent evidence suggests that cardiomyocytes might also produce endothelin-1, which might directly impair myocyte contractility by increasing intracellular calcium levels. Because hyperglycemia is a potent stimulus of endothelin-1 production, we hypothesized that increased production, action, or both of endothelin-1 might be a mediator of direct cardiomyocyte injury in diabetes. Therefore we studied the effects of endothelin receptor blockers (BQ-123 and bosentan) on hyperglycemia-induced endothelin-1 production and cellular injury after ischemia-reperfusion.

Methods: Using a human ventricular heart cell model of simulated ischemia-reperfusion, we studied the effects of normoglycemia (5 mmol/L, 48 hours) and hyperglycemia (25 mmol/L, 48 hours) on cellular injury and endothelin-1 production. Furthermore, the effects of selective endothelin-A and mixed endothelin-A/B receptor antagonism (with BQ-123 and bosentan, respectively) were evaluated.

Results: Cellular injury, as assessed by means of trypan blue uptake, was higher in human ventricular heart cells subjected to hyperglycemia and simulated ischemia-reperfusion injury ($P = .01$); this effect was prevented with both BQ-123 and bosentan ($P = .01$). In addition, heart cells from the hyperglycemic group elaborated more endothelin-1 after ischemia-reperfusion ($P = .02$).

Conclusions: Endothelin-1 production and cellular injury were greater in human ventricular heart cells subjected to hyperglycemic conditions and simulated ischemia-reperfusion. These effects are mediated by endothelin-A receptors because both BQ-123 and bosentan exerted similar degrees of protection. Endothelin receptor blockade is a novel strategy to improve the resistance of the diabetic heart to cardioplegic arrest and reperfusion.

W̱e have previously demonstrated an important vasoconstricting response mediated by endothelin-1 (ET-1) during reperfusion injury in diabetes.[1] In this study the coronary effluent release of ET-1 was higher in diabetic than in nondiabetic patients after cardiopulmonary bypass (CPB) and reperfusion. Furthermore, diabetic coronary microvessels responded to CPB and reperfusion with greater ET-1-mediated vasoconstriction and

diminished nitric oxide-mediated vasodilatation; these effects were attenuated by ET antagonism.[1]

Recent evidence suggests that cardiomyocytes (in addition to endothelial cells) might also produce ET-1, which might directly impair myocyte contractility by increasing intracellular calcium levels.[2] In the present study we hypothesized that hyperglycemia directly impairs cardiomyocyte survival through the production of ET-1. To this aim, we examined the effects of ET receptor blockade (with BQ-123 and bosentan) on ET-1 production and cellular injury in human cardiomyocytes subjected to simulated ischemia-reperfusion in hyperglycemic compared with normoglycemic environments.

Patients and Methods

Human Heart Cell Model of Ischemia-Reperfusion

Our method of culturing heart cells from human ventricular biopsy specimens has been previously described in detail.[3-5] In brief, 5- to 20-mg biopsy specimens were obtained from the right ventricular outflow tracts of patients undergoing elective surgery for tetralogy of Fallot. After digestion with trypsin (0.2%) and collagenase (0.1%), the separated cells were seeded onto cell-culture dishes and cultured at 37°C in a 5% carbon dioxide atmosphere in Iscove's modified Dulbecco's medium with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.1 mmol/L β-mercapthoethanol. Cells were cultured under either hyperglycemic (25 mmol/L) or normoglycemic (5 mmol/L) conditions for 48 hours. Purification was achieved by using a dilution cloning technique. Enzymatically isolated cells were seeded at a low density to enable morphologic identification of individual cardiomyocytes and separation from contaminating cell types. Single cardiomyocyte colonies were then transferred to a separate culture dish. The use of cardiomyocytes facilitates examining the effects of ischemia-reperfusion independent of other cell types, such as endothelial cells or fibroblasts. Our technique of simulated ischemia-reperfusion has also been described in detail previously.[3,4] In brief, after 30 minutes of stabilization in 10 mL of normoxic phosphate-buffered saline solution (PBS), ischemia was simulated by exposing the cells to a low volume (1.6 mL) of anoxic PBS at 37°C for 90 minutes. During this period, the cells were placed in an airtight Plexiglas chamber, which was continuously flushed with 100% nitrogen to maintain anoxic conditions. Ischemia was followed by a reperfusion period in which the cells were exposed to 10 mL of normoxic PBS at 37°C for 30 minutes. Both low volume and anoxic conditions were used to mimic ischemia. The volume of anoxic perfusate used (1.6 mL) was the minimum volume required to coat the cellular monolayer for the prevention of cellular dehydration during the ischemic period. To verify the presence of anoxia, we placed 2 mL of anoxic PBS in a center dish within the sealed chamber and tested at the termination of each ischemic period to ensure a Po_2 of 0 mm Hg. Anoxic PBS was prepared by bubbling with 5% carbon dioxide and 95% nitrogen that had first been passed through an oxygen trap (1% wt/vol $NaHSO_3$ in deionized water). The solution (monitored with a blood gas analyzer) was degassed until a measured Po_2 of 0 mm Hg was achieved. The pH was adjusted to 7.4 $\pm$ 0.05, and an osmolality of 290 $\pm$ 20 mOsm/L was ensured before use. For the

assessment of cellular injury, cells were stained with trypan blue at the end of the reperfusion period (ie, after 150 minutes of incubation for the nonischemic groups). Injured cells were unable to exclude the large-molecular-weight dye and stained blue. The above protocol was repeated in the presence of BQ-123 (ET$_A$ receptor blocker, 1 μmol/L) and bosentan (ET$_{A/B}$ receptor blocker, 100 μmol/L; Actelion Ltd) added before reperfusion. A single blinded observer performed cell counts. In addition, we measured ET-1 production from human heart cells after ischemia-reperfusion in the hyperglycemic and normoglycemic conditions. Heart cells were lysed with NP40, after which a commercial enzyme immunoassay kit was used to assess ET-1 production (American Research Products, Inc).

Statistical Analysis

Data are presented as means $\pm$ SEM. Data were compared with a 2-way analysis of variance. When the F ratio indicated a significant effect, differences were specified by using a Newman-Keuls test for post hoc comparisons.

Results

Effects of Hyperglycemia Versus Normoglycemia on Human Heart Cell Injury

Figure 1 depicts the effects on cellular injury of culturing human ventricular heart cells with either 5 or 25 mmol/L glucose for 48 hours, as assessed with trypan blue exclusion. Heart cells subjected to simulated anoxia and reoxygenation exhibited greater cellular injury (cell survival of 52% $\pm$ 4% vs 28% $\pm$ 3% for normoglycemic conditions, P = .01). Importantly, this effect was prevented by the ET$_A$ specific antagonist BQ-123 and the mixed ET$_{A/B}$ antagonist bosentan (when administered before reperfusion, Figure 1). ET blockade did not affect cell survival in the nonischemic group (cell survival of nonischemic + bosentan [normoglycemia] group of 22% $\pm$ 3% vs 17% $\pm$ 4% for nonischemic group; P = .33; cell survival of nonischemic + bosentan [hyperglycemia] group of 18% $\pm$ 4% vs 20% $\pm$ 3% for nonischemic group; P = .40).

Hyperglycemia Exaggerates ET-1 Production

The effects of simulated hyperglycemia on ET-1 production were studied. Hyperglycemic heart cells produced greater amounts of ET-1 after simulated ischemia-reperfusion when compared with those in the normoglycemic group (418 $\pm$ 45 vs 303 $\pm$ 23 in the normoglycemic group, P = .02; Figure 2).

Discussion

We hypothesized that hyperglycemia might stimulate heart cells to produce ET-1, which might increase cell injury after simulated ischemia-reperfusion. Our data demonstrate that human ventricular heart cells subjected to 48 hours of hyperglycemia elaborated more ET-1 and had less cell survival. Furthermore, BQ-123 and bosentan prevent hyperglycemia-induced cell injury when used before reperfusion.
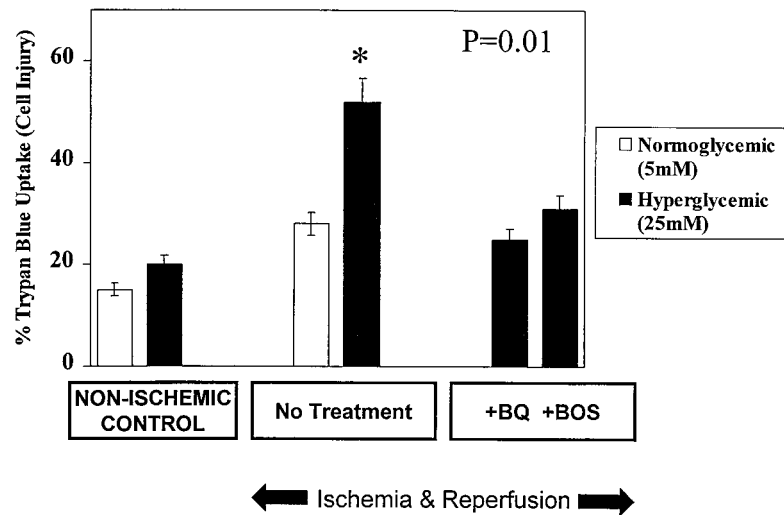
Figure 1. Cellular injury (assessed by means of trypan blue uptake) in human ventricular heart cells subjected to simulated ischemia-reperfusion. Cells subjected to 48 hours of hyperglycemia exhibited increased cellular damage. This response was prevented by BQ-123 (an ET_A antagonist) and bosentan (an $ET_{A/B}$ antagonist). *P = .01 versus normoglycemic group and hyperglycemic group plus BQ-123 and hyperglycemic group plus bosentan.
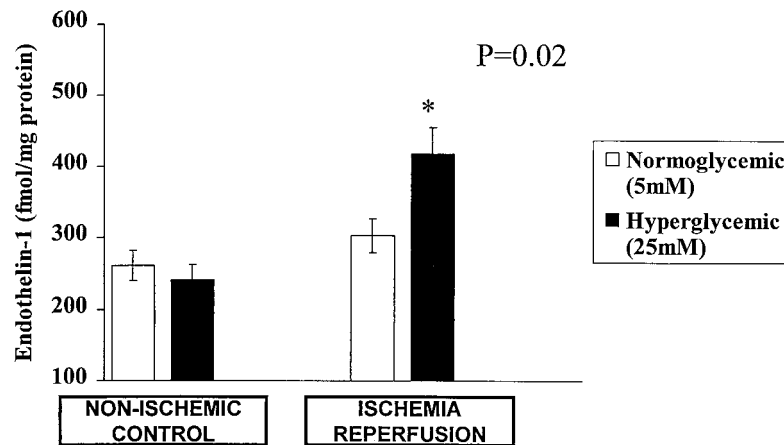

Figure 2. Effects of normoglycemia versus hyperglycemia on human heart cell ET-1 production. Hyperglycemic heart cells elaborate more ET-1 after ischemia-reperfusion when compared with those in the normoglycemic group. *P = .02 versus normoglycemic group.

These data suggest, for the first time, an effect of ET-1 on human heart cell injury independent of other cell types, such as endothelial cells, platelets, or neutrophils. Dorman et al[2] have demonstrated that myocyte ET-1 exposure during cardioplegic arrest exacerbates contractile dysfunction after reperfusion by mean of alterations in intracellular calcium homeostasis and a reduction in β-adrenoceptor responsiveness, a phenomenon that frequently occurs after cardiac operations. Hyperglycemia in diabetic patients might impair contractility through these mechanisms. We have previously demonstrated that diabetes is an independent predictor

of low output syndrome after coronary artery bypass grafting,[6] and hence it is tempting to speculate that increased myocyte ET-1 production might be an important contributor to this phenomenon.

Our model of ischemia-reperfusion does not completely reproduce the in vivo responses. The heart cells used in these studies have been extensively evaluated in previous reports. These myocytes retain many characteristics of freshly isolated cells but have distinct differences. These cells become quiescent after enzymatic digestion and passaging. Despite an abundant supply of mitochondria and

contractile proteins, the sarcomeres become disrupted during division and do not reestablish their characteristic functional format. However, the metabolic response of these cells after ischemia is similar to our intraoperative findings during cardiac operations.[7-9] Furthermore, the cellular concentrations of troponin I, troponin T, and creatine kinase MB isoform are similar to those seen in vivo. Although the molecular and biochemical characteristics of these cells resemble in vivo cardiomyocytes, these cells undergo phenotypic changes, become quiescent, and regain their ability to divide. Therefore these cells retain many characteristics of normal human myocardium and might simulate the human heart during cardioplegic arrest. The heart cells in culture are easily differentiated from other cell types. Endothelial cells are oval (15 × 20 mm) and fibroblasts are spindle-shaped (4 × 80 mm) compared with the rectangular and much larger heart cells (40 × 80 mm). In addition, endothelial cells grow poorly in the medium used for heart cells, whereas fibroblasts have a much faster doubling time in culture and are easily identified as a spindle-shaped contaminant. Our model of ischemia-reperfusion is similar to the effects of global ischemia on the myocardium. Although the volume overlying our cells during ischemia exceeds the volume of fluid surrounding cardiomyocytes in the globally ischemic heart, the reduction of the volume of ischemic PBS from 10 to 1.5 mL resulted in a marked increase in the products of ischemic metabolism (lactate), a decrease in extracellular pH, and progressive cell injury. Thus our model simulates low-flow ischemia analogous to limited cardioplegic perfusion during cardiac operations.

The addition of glucose to cardioplegic solutions was demonstrated to be detrimental if the products of anaerobic metabolism (lactate acidosis) were not washed from the heart with repeated cardioplegic infusions. Because acidosis might increase ET-1 production, the detrimental effects of glucose might be due to excessive ET-1 production.

Clinical Perspective

There is a growing body of evidence to suggest that ET-1 might represent an important mediator of perioperative injury during cardiac operations.[10-15] Elegant studies by Bond et al[10] and Ergul et al[15] have suggested that exaggerated ET-1 production during CPB is closely associated with indices of postoperative recovery, including prolonged intensive care unit stay, ventilation times, and inotropic support. Furthermore, exaggerated ET-1 production during cardiac operations might represent an important mediator of perioperative vasospasm in conduits used for bypass grafting.[15] In addition, increased production of ET-1 by the heart and pulmonary circulation might be one of the key determinants of increased pulmonary arterial pressure noted postoperatively.[10-15] Importantly, antagonism of ET-1 action with potent ET receptor antagonists has been demonstrated to prevent CPB-induced increases in pulmonary

vascular resistance.[12] The work presented in our articles extends this body of evidence and suggests that the diabetic milieu exaggerates the deleterious effects of ET-1 on vascular reactivity and myocyte survival. Given the increasing understanding of the role of ET-1 in cardiac surgery, it is reasonable to suggest that a phase I study be conducted to develop the use of ET receptor antagonists, such as bosentan, for myocardial protection in high-risk patients undergoing cardiac surgery.

Conclusion

ET-1 production and cellular injury was amplified when human ventricular heart cells were subjected to hyperglycemia before simulated ischemia-reperfusion. However, both ET-1 production and cell death were reduced by means of treatment with BQ-123 and bosentan before reperfusion. These observations, in conjunction with those made in our previous publication,[1] suggest that antagonism of ET receptors might decrease endothelial dysfunction and reduce cardiomyocyte death in diabetes and hyperglycemic conditions. Diabetic hyperglycemia might exacerbate reperfusion injury in ischemic regions being revascularized, and bosentan might represent a novel therapy for the cardiovascular complications of diabetes.[16,17]

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Discussion

Dr Alain Carpentier *(Paris, France).* I enjoyed your presentation very much. I think it is a very important one. I would like to ask you one question. You are talking about cardioplegia. As you know, there are different sorts of cardioplegia, particularly blood cardioplegia and crystalloid cardioplegia. Have you investigated whether these might have some effect and whether it is the cardioplegic arrest or the type of substance used to carry out cardioplegia that might have an influence?

Dr Verma. Thank you very much for your comments.

This was a small study, and all of our patients were subjected to one particular type of cardioplegia, blood cardioplegia. We have not compared that with crystalloid cardioplegia. I do believe that Dr Sellke's group has demonstrated some very nice differences between crystalloid cardioplegia and blood cardioplegia in terms of atrial microvessel and endothelial function. But this certainly needs to be followed up, and I thank you for your question.

Dr Jakob Vinten-Johansen *(Atlanta, Ga).* That was a very nice study, and I think future studies certainly will point toward ET as a contributor to ischemia-reperfusion injury.

Do you think that the effects of ET are simply acting in opposition to nitric oxide in terms of its vasoreactivity responses, or is it actually playing an active role in initiating and promulgating the reperfusion injury response? For example, does it stimulate the release of proinflammatory mediators, does it stimulate the upregulation of adhesion molecules, or does it stimulate neutrophils themselves?

Dr Verma. You bring up an interesting point. First, you highlight one of the limitations of the study, and that is that we have not provided an index of nitric oxide production. Therefore it is hard to understand what the regulation between ET and nitric oxide is in this model. However, there are recent studies by Dr Sharma's group that demonstrate that diabetic patients might elaborate greater amounts of ET in the face of normal nitric oxide production.

Your question about the other effects of ET and how it might relate to the tenets of ischemia-reperfusion is also an important one. We do not have evidence on other mechanisms; however, ET has now been implicated as an important mediator of P-selectin-dependent neutrophil rolling and later firm adhesion of the neutrophils.

Professor Magdi Yacoub *(London, United Kingdom).* I have 2 questions. One is that you have shown hyperreactivity to ET. Would you like to speculate or have you investigated why that is? Is it increased numbers of receptors?

The second question is, ET is not all bad news, as you know, because it is a compensatory thing, and it has an inotropic effect. Therefore would you like to tell us why is it that the body is doing this in the first place in diabetes? Is it trying to do something that you are trying to block?

Dr Verma. Thank you for your comments, Professor Yacoub. In response to your first question, the fact that the responsiveness to ET in the microvasculature is enhanced, what are the subcellular mechanisms mediating that effect? When you think of this, the first thing that would come to mind is, are the ET receptor densities different? In diabetic experimental models, ET receptor density remains the same. Therefore it is probably an event that is below the ET receptors and might involve greater sensitivity of the myofibrils to the effects of ET.

Now, in response to your second question—this homeostatic mechanism—as to why ET goes up, is it a compensation? ET, as you mentioned, might be a compensatory positive inotrope. That appears to not be true in diabetic experimental models of heart failure. The positive inotropic effects of ET might occur in a noncardiomyopathic or a noncongestive heart failure type of model as opposed to a failing heart.

I thank you very much for your comments.