Research

Insulin-like growth factor-1 protects ischemic murine myocardium from ischemia/reperfusion associated injury

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Abstract

Introduction Ischemia/reperfusion occurs in myocardial infarction, cardiac dysfunction during sepsis, cardiac transplantation and coronary artery bypass grafting, and results in injury to the myocardium. Although reperfusion injury is related to the nature and duration of ischemia, it is also a separate entity that may jeopardize viable cells and ultimately may impair cardiac performance once ischemia is resolved and the organ heals.

Method The present study was conducted in an ex vivo murine model of myocardial ischemia/reperfusion injury. After 20 min of ischemia, isolated hearts were perfused for up to 2 hours with solution (modified Kreb’s) only, solution plus insulin-like growth factor (IGF)-1, or solution plus tumor necrosis factor (TNF)-α. Cardiac contractility was monitored continuously during this period of reperfusion.

Results On the basis of histologic evidence, IGF-1 prevented reperfusion injury as compared with TNF-α; TNF-α increased perivascular interstitial edema and disrupted tissue lattice integrity, whereas IGF-1 maintained myocardial cellular integrity and did not increase edema. Also, there was a significant reduction in detectable creatine phosphokinase in the perfusate from IGF-1 treated hearts. By recording transduced pressures generated during the cardiac cycle, reperfusion with IGF-1 was accompanied by markedly improved cardiac performance as compared with reperfusion with TNF-α or modified Kreb’s solution only. The histologic and functional improvement generated by IGF-1 was characterized by maintenance of the ratio of mitochondrial to nuclear DNA within heart tissue.

Conclusion We conclude that IGF-1 protects ischemic myocardium from further reperfusion injury, and that this may involve mitochondria-dependent mechanisms.

Keywords apoptosis, mitochondrial DNA, myocardium, reperfusion injury, sepsis

ANOVA = analysis of variance; CPK = creatine phosphokinase; IGF = insulin-like growth factor; MK = modified Kreb’s Henseleit working solution; mtDNA = mitochondrial DNA; nDNA = nuclear DNA; PCR = polymerase chain reaction; PI3 = phosphatidylinositol-3; ΔPsys/dia = difference between ex vivo systolic and diastolic pressure; ROS = reactive oxygen species; TNF = tumor necrosis factor; VEGF = vascular endothelial growth factor.
Introduction

Cardiovascular diseases are among the leading causes of death in North America. The most important presentation of cardiovascular disease is ischemia, which leads to tissue hypoxia, cellular necrosis, apoptosis and, in severe situations, organ dysfunction. The main treatment for acute ischemic heart disease is early vascular reperfusion to restore balance to cardiac metabolic demands. Although reperfusion is the foundation of therapy, it may actually initiate further injury to the myocardium. Although the phenomenon of reperfusion injury is related to the duration of ischemia, it is a separate entity and may be more severe than ischemic injury alone [1,2]. Ischemia/reperfusion injury can be generated in various cardiovascular diseases/events or therapies, including myocardial infarction, cardiopulmonary bypass, coronary bypass grafting, heart transplantation, and coronary thrombolytic therapy. It has also been speculated that the mechanism of myocardial dysfunction during septic shock is related to segmental ischemia and reperfusion in the left ventricular wall, because the involvement of persistent global ischemia has been disproved [3,4].

Ischemia results from the absence of or sluggish blood flow in coronary vessels. This leads to a mismatch between cardiac metabolic supply and demand. Ischemia of short duration may contribute to ‘stunned myocardium’ without tissue injury, but prolonged ischemia results in a deficiency in energy supplies and waste removal, with eventual initiation of myocardial ischemia and reperfusion in the left ventricular wall, because the involvement of persistent global ischemia has been disproved [3,4].

Reperfusion injury is mediated by inflammation and characterized by the production of reactive oxygen species (ROS). Production of ROS may be initiated during the ischemic phase, generating ‘primed’ myocardium. ROS activate transcription factors, such as nuclear factor-κB, both in cardiac myocytes and the endothelium; in turn, this initiates transcription of genes including those encoding adhesion molecules, cytokines, coagulation mediators, and proteolytic enzymes [6]. In coordination with the complement cascade, ROS can disrupt the integrity of both cardiac myocyte and endothelial cell membranes [7]. These events can change intracellular ion homeostasis, resulting in the accumulation of calcium and metabolic byproducts. These changes increase the activation of enzymes that are utilized in the processes of necrosis and apoptosis, and that alter mitochondrial function [8]. At the tissue level, this is manifested by interstitial edema and disruption of the tissue lattice. Concomitantly, neutrophils and other inflammatory cells migrate into the injured zone using adhesion molecules such as intercellular adhesion molecule-1 under the stimulation of secreted cytokines and chemotactic factors. Recruitment and infiltration of neutrophils into the injured tissue is accompanied by neutrophil degranulation and further injury to the border zone of viable cells. These late cellular events in the myocardium only occur after reperfusion [2,5,9,10].

Sustainable functioning of the myocardium is the central objective of therapeutic intervention in myocardial infarction. Cardiac function and contractility are closely related to cardiac metabolism and energy production. In cardiomyocytes energy production is related to the number of mitochondria, with these organelles occupying up to 40% of the cardiomyocyte cytoplasm. Hence, the total number of mitochondria in the myocardial tissue can be used as a measure of cardiomyocyte activity and health [11,12]. In HIV-infected patients with symptomatic hyperlactatemia receiving antiretroviral therapy, Cote and coworkers [13] showed that the ratio of mitochondrial DNA (mtDNA) to nuclear DNA (nDNA) can be used as a marker of drug-induced mitochondrial toxicity. During the past century, there have been major improvements in the strategies used to protect myocardial tissue from ischemia/reperfusion injury [14–18]. However, the mechanism of ischemia/reperfusion injury remains unknown, and our abilities to treat and prevent it are therefore limited. Using methods similar to those used by Cote and coworkers [13], we investigated changes in heart mtDNA : nDNA ratio during myocardial ischemia and reperfusion phases, and compared these levels with additional measures of tissue injury.

New markers of myocardial injury may provide mechanistic insights and reveal therapeutic possibilities in reperfusion injury. Here, we propose a new method, using insulin-like growth factor (IGF)-1, of protecting cardiac tissue against ischemia/reperfusion injury in an ex vivo murine model. The mechanism underlying this protective effect remains unclear. However, the integrity of the myocardial tissue lattice was preserved and the development of interstitial edema in myocardial tissue was inhibited. These effects were correlated with improved perfusion pressure and left ventricular compliance. We also demonstrated that this IGF-1 mediated protection was accompanied by preservation of mtDNA content. The relevance of this finding to tissue function is discussed.

Methods

Ischemia/reperfusion model

C57B6 mice (Jackson Laboratories, Bar Harbor, ME, USA), weighing 25–30g, were anesthetized using 3% isoflurane (Baxter, Toronto, Ontario, Canada) for 1 min and maintained with 1% isoflurane for 3–5 min during cardiac excision. To prevent coagulation in coronary vessels, 500 U heparin–sodium (Organon Teknika Inc., Toronto, Ontario, Canada) was injected intraperitoneally 10 min before induction of anesthesia. The heart was excised and assembled on a Langendorff apparatus and perfused with oxygenated (95% oxygen, 5% carbon dioxide), modified Kreb’s Henseleit working solution (MK) at 37°C for 3–5 min [19–21] until monitoring revealed that the organ was stable. Transduced left
ventricular and aortic pressures, and heart rate were monitored continuously using Power lab/8sp detectors (AD Instruments Pty Ltd., Castle Hill, Australia). Retrograde perfusion was then stopped for 20 min to model global ischemia (a period of 20 min of ischemia was found to be optimal for the ischemic phase in this model). The ischemic hearts were then reperfused with MK solution alone, MK solution plus IGF-1 during the ischemic phase in this model. The ischemic hearts were then stopped for 20 min to model global ischemia (a period of 20 min of ischemia was found to be optimal for the ischemic phase in this model). The ischemic hearts were then reperfused with MK solution alone, MK solution plus IGF-1 (10 ng/ml) for 1 or 2 hours. After completion of the reperfusion period, the hearts were divided into halves. One half was frozen using 2-methylbutane (Isopentane; MERK KgaA, Darmstadt, Germany) in liquid nitrogen for 80 s for eventual sectioning and/or DNA isolation. Paraffin embedding of the other half followed fixation of the sample in 10% formalin.

**Histologic evaluation of different groups**

Slides of paraffin embedded tissues from the apex to the basal portion of the hearts were prepared and stained with hematoxylin and eosin. Serial 400× magnified images were captured using a Nikon E600 microscope and Spot Advanced software (version 3.4.2; S. Leffler & Silicon Graphics Inc., Mountain View, CA, USA). Image Pro-Plus software (MediaCybernetics, Carlsbad, CA, USA) was used to evaluate the severity of interstitial edema around the perivascular spaces of coronary arteries and veins. Measures were taken in 10 sections from each of four hearts for all conditions and time points. The extent of interstitial edema was measured by selecting a circular area with a radius two times greater than the vascular space contained within the drawn circle. The total vascular and perivascular areas were measured. ‘Nontissue’ area was determined by color segmentation images constructed by Image Pro-Plus. Total interstitial edema was determined by subtracting the vascular area from the nontissue area and expressing this as a percentage of total perivascular area: percentage edema = [(nontissue area – total vascular area)/total perivascular area] × 100%.

**Ventricular function assessment**

Pressure generated during the cardiac cycle was obtained by transduction of the aortic cannula and recorded continuously during ischemia and reperfusion using a Power lab/8sp detector. Using Powerlab software, the difference between ex vivo systolic and diastolic pressure ($\Delta P_{sys/dia}$) at different time points was calculated to assess ventricular performance. Pressure measured during systole reflected contractility, and diastolic pressure drops reflected relaxation of the ventricle. Thus, greater $\Delta P_{sys/dia}$ values indicate better overall performance of the left ventricle.

**Detection of creatine phosphokinase**

A 1 ml sample of myocardial perfusate was collected every 15 min during the reperfusion phase. The samples were frozen in a mixture of ethanol and dry ice [22,23]. The level of creatine phosphokinase (CPK) was measured using Vitros CK slides (Ortho-Clinical Diagnostics, Rochester, NY, USA). Briefly, 11 μl perfusate was deposited on the slide and evenly distributed. Samples were incubated for 5 min at 37°C. After final interaction, leuco-dye is oxidized by hydrogen peroxide in the presence of peroxidase to form an insoluble dye. Reflection densities are monitored during incubation, and the rate of change in reflection density is then converted to enzyme activity by using 670 nm wavelength in the Vitros Chemistry 250 System.

**Mitochondrial/nuclear DNA assay**

Frozen hearts embedded in opaque tissue fixation material were thawed, cut into small pieces (approximately 3 mg), and then placed into lysis buffer. DNA was extracted using the Qiagen DNA isolation kit (Qiagen Canada, Qiagen Inc., Mississauga, Ontario, Canada), in accordance with the manufacturer’s protocol. Extracts were then diluted 1:80 with buffer AE before performing the mtDNA assay, as reported previously [11,13,24] but modified for application in murine tissues as described below.

For each DNA extract, one murine nuclear gene (accessory subunit of the murine mitochondrial DNA polymerase γ (ASPG); Genbank accession number AF177202) and one murine mitochondrial gene (cytochrome oxidase subunit 1 (COX), Genbank accession number AB042432) were quantified separately with real-time, quantitative PCR, using the Roche LightCycler (Roche Diagnostics, Indianapolis, IN, USA). For the mitochondrial (COX) gene, the forward primer mCOX1F (5′-TCGTGATTATCTCACAACCAATCA-3′) and the reverse primer mCOX2R (5′-GCCTCCAATTATTGGTATTTACTATGA-3′) were used. The oligonucleotides 3′-fluorescein-mCOXPR1 (5′-AACCCAGTGCACTTTTAGGAGATGC-3′) and the reverse primer mCOX2R (5′-GCCTCCAATTATTGGTATTTACTATGA-3′) were used as hybridization probes. For the nuclear (ASPG) gene, the forward primer mASPG1F (5′-GGAGGAGGCCACTTTTGCACC-3′) and 5′-LC Red 640 3′-phosphate-blocked-mCOXPR2 (5′-AAATTACAATGTATCGTAACACTGCCCATGC-P3′) were used as hybridization probes. The same standard curve variations were under 5%.

A standard curve consisting of serially diluted mouse DNA (30,000, 6000, 1200, 240 and 48 nuclear genome equivalents) were included in each run. The same standard curve was used to quantify both the nuclear (ASPG) and the mitochondrial (COX) genes. mtDNA and nDNA genes were assayed in duplicate. Results of the quantitative PCR assay were expressed as the ratio of the mean value of the duplicate mtDNA measurements to the mean value of duplicate nDNA measurements. As a further quality control, a mouse DNA extract with a mtDNA:nDNA ratio known to be high, and an extract with a mtDNA:nDNA ratio known to be low were included in every run. Repeat sample and intrasample variations were under 5%.
Statistical analysis
Values are expressed as mean ± standard error. P<0.05 was considered statistically significant.

Results
Perivasular interstitial edema and tissue lattice integrity
The cellular integrity of the myocardium was well preserved in the tissue of hearts reperfused with IGF-1 (Fig. 1). The area of interstitial edema in hearts treated with IGF-1 plus MK was 21 ± 4%, as compared with 34 ± 6% and 49 ± 5% for reperfusions with MK only and MK plus TNF-α, respectively. Representative tissue histology images are presented in Fig. 1 and were similar throughout the four hearts and in all conditions. Additional histology observations included an increased number of shrunken, contracted myocytes with dense pyknotic nuclei with MK plus TNF-α reperfusion as compared with perfusate containing IGF-1. Using single factor analysis of variance (ANOVA), the differences in percentage edema between groups were statistically significant (P<0.05).

Insulin-like growth factor-1: improvement in myocardial performance during reperfusion
Cardiac performance was determined by calculating the pressure difference between systole and diastole (i.e. ∆P_sys/dia) at set time points. The systolic and diastolic pressures were determined by taking the average values from a window around the respective time points. Performance is then a measure of both contractility (stroke volume and force of left ventricular contraction, manifesting as systolic pressure) and diastolic function, or relaxation of the left ventricle (a reduction in diastolic pressures). Improved performance is manifested by a widening in ∆P_sys/dia. Pressure monitoring demonstrated that cardiac performance increased from 0 to 40 min of reperfusion for all conditions (Fig. 2). After 40 min of reperfusion, cardiac performance arrived at a plateau and became negative for the remaining minutes for the MK alone and the MK plus TNF-α reperfusions. With reperfusion with MK plus TNF-α the difference between systolic and diastolic pressure (∆P_sys/dia) initially increased to 6.8 ± 0.7 mmHg as compared with reperfusion with MK alone (5.1 ± 0.6 mmHg). However, reperfusion with IGF-1 generated a ∆P_sys/dia that was significantly greater (13.8 ± 1.2 mmHg) than that with TNF-α (6.8 ± 0.7 mmHg) by 20 min. This gain in cardiac performance was maintained up to 120 min of reperfusion with IGF-1. The enhanced performance was reflected in improvements in both systolic and diastolic pressures. The late descent in slope at 120 min of reperfusion with IGF-1 was similar to that occurring with reperfusion with MK alone and with MK plus TNF-α, but may relate to ex vivo conditions other than the ischemia time and the reperfusion solution. A paired, two sample t-test for means between groups demonstrated a statistically significant difference between IGF-1 and MK alone and MK plus TNF-α (P<0.005).

Low creatine phosphokinase level in insulin-like growth factor-1 treated hearts
Collected perfusate from hearts treated with IGF-1, at all reperfusion time points, contained significantly lower quantities of detectable CPK (34.6 U/l) than did perfusate from TNF-α treated hearts (113.6 U/l). This is shown as an average for all time points in Fig. 3. Single factor ANOVA revealed a statistically significant difference between groups (P<0.005).

Ratio of mitochondrial to nuclear DNA
IGF-1 maintained or improved the mtDNA:nDNA ratio during reperfusion of ischemic myocardium as compared with control reperfusion with MK alone. There was a significant difference between all test groups (baseline, ischemia, reperfusion with modified Kreb’s Henseleit working solution (MK) alone, MK plus tumour necrosis factor (TNF)-α, and MK plus insulin-like growth factor (IGF)-1, both at 1 and 2 hours. Note the preservation of cellular and structural elements and the lack of interstitial edema in the IGF-1 reperfused heart. Magnification for all images: 400×.
Davani et al. (ischemia) followed by subsequent reperfusion (Fig. 4). Such a reduction was noted with reperfusion with MK alone after the initial increase in mtDNA:nDNA ratio that occurred after ischemia alone without reperfusion. Because this model utilized a cell-free perfusate, the mtDNA:nDNA ratio is not confounded by potential contributions from immune cells – a point that has been raised as a possible explanation for changes in mtDNA:nDNA ratio.

**Discussion**

Despite a range of clinical interventions, our ability to prevent reperfusion injury after disruption of blood flow to vascular
beds remains disappointing. An appreciation of the mechanism of ischemia/reperfusion injury is central to development of better treatments. In the present study we demonstrated that IGF-1 can lessen reperfusion injury following an initial ischemic insult.

This effect of IGF-1 on the ischemic myocardium was supported by histologic evidence of improved tissue and cellular integrity, including markedly less interstitial edema around the perivascular spaces. In this model, ischemic myocardium treated with IGF-1 had significantly lesser amounts of detectable CPK than did myocardium treated with TNF-α, suggesting reduced cellular injury. This is also consistent with the cardiac performance and left ventricular contractility of IGF-1 treated hearts, that exhibited a greater ΔP_sys/dia. It should be noted that the detectable CPK levels would not be above the normal range as determined for human whole blood samples. However, there was considerable histologic evidence of tissue damage in the TNF-α treated hearts, suggesting the relative insensitivity of CPK in detecting lesser myocardial injuries. A more sensitive marker would be valuable not only for studying the mechanism that underlies reperfusion injury but also for evaluating the efficacy of therapeutic interventions. This is particularly true when one considers the segmental and intermittent ischemic/reperfusion zones that characterize dysfunctional myocardium in sepsis. The initial improvement in contractility, observed under all conditions of reperfusion, was probably the result of a new supply of nutrients after the ischemic period, including oxygen. The addition of IGF-1 significantly augmented this improvement in left ventricular pressure generation and relaxation (thus increasing ΔP_sys/dia). This improvement was maintained throughout the period of reperfusion.

Myocardial performance at the cellular level is associated with the number or functional capacity of mitochondria. To investigate indirectly whether mitochondrial function may represent a marker of this beneficial effect of IGF-1, we determined the mtDNA : nDNA ratio in relation to myocardial function. Although not appreciated clinically, ischemia and reperfusion are two distinct periods [10,14,16,25,26]. The ischemic period has been described as ‘priming’ cardiac myocytes for either necrotic or apoptotic death. A marked increase in mtDNA : nDNA ratio was detected in the ischemic myocardium relative to baseline control levels. Apoptosis has been found to be an event that requires energy [27,28]. Whether this increased mtDNA : nDNA ratio indicates an increase in the number of mitochondria per cell or an increase in the genome copy number per mitochondria remains to be determined.

Myocardial reperfusion injury, as a separate event, can increase the extent of injury beyond that caused by ischemia alone. It has been shown that modification of solutions or other conditions during the reperfusion phase can alter the extent of cellular and functional damage to the myocardium. We determined that the nature of the reperfusate can affect mtDNA : nDNA ratio. Reperfusion with MK alone resulted in a reduction in mtDNA : nDNA ratio toward baseline values. This may reflect either mitochondrial mitoptosis in damaged and ‘primed’ tissues, or necrotic loss of similar cells that were ‘primed’, resulting in elevated mtDNA : nDNA ratio after ischemia. The net effect would be that the remaining tissue is spared and should reflect baseline tissue. However, a mtDNA : nDNA ratio that does not differ from baseline does not indicate that the tissue is working normally. In fact, histology and contractility determinations demonstrated that the heart had sustained significant tissue damage and was dysfunctional after MK reperfusion. With IGF-1 reperfusion this reduction in mtDNA : nDNA ratio was prevented, suggesting that the extent of injury is not associated with elevated mtDNA : nDNA ratio alone. In fact, after ischemia/reperfusion, it was found that a normal mtDNA : nDNA ratio early after reperfusion predicted significant tissue injury. The patterns of mtDNA : nDNA ratio, as seen in this model, may prove useful in future investigations of possible mitochondria-related mechanisms of reperfusion injury.

IGF-1 can affect cardiomyocyte contractility through its receptor – a heterotetrameric protein with intracellular tyrosine kinase activity [29]. Downstream signals after receptor activation include Shc, Crk and phospholipase C, and activation of phosphatidylinositol-3 (PI3) kinase. Guse and coworkers [30] demonstrated that IGF-1 can increase PI3 levels in rat cardiomyocytes. Through its action on PI3 kinase, IGF-1 can affect both contractility [31] and apoptosis [32]. The action of IGF-1, as demonstrated in our myocardial ischemia/reperfusion model, may occur via PI3 kinase and/or effects on mitochondria. Increases in cardiomyocyte calcium levels and cardiomyocyte sensitivity to calcium [33] have been demonstrated to effect cardiac performance. Alteration in calcium metabolism may interfere with the action of calcium because the filamentous network of cardiomyocytes and their contractile properties are extremely sensitive to even small fluctuations in calcium ion concentration [34].

A similar result to that presented here for IGF-1 in myocardial ischemia/reperfusion has been demonstrated for vascular endothelial growth factor (VEGF), suggesting that a final common ‘protective’ pathway may exist [35]. Anwar and coworkers [36] showed that TNF-α decreased IGF-1 mRNA and increased IGF-1 binding protein-3 mRNA expression in vascular smooth muscle cells. These actions of TNF-α effectually reduced free IGF-1 levels and activity, and promoted endothelial instability. Infusion of a modified IGF-1 reduced the TNF-α induced apoptosis. An interaction between VEGF and IGF-1 was characterized in retinal neovascularization in diabetic patients [37]. The authors of that report described common mitogen-activated protein kinase 44/42 pathways that may be related to the mitogenic effect of those two molecules. However, the short time to effect for both IGF-1 and VEGF in myocardial ischemia/reperfusion models is most
In an *ex vivo* model of myocardial ischemia and reperfusion, IGF-1 protects against reperfusion-associated injury and improves cardiac performance. This protective effect correlates to mtDNA: nDNA ratio that was elevated with respect to baseline and may represent a marker for the preservation of mitochondrial function. This study provides new insight into ischemia reperfusion and possible mechanisms and treatment for the tissue injury and organ dysfunction that is associated with this process.

**Key messages**

- In an *ex vivo* model of myocardial ischemia and reperfusion, IGF-1 protects against reperfusion-associated injury and improves cardiac performance.
- This protective effect correlates to mtDNA:nDNA ratio that was elevated with respect to baseline and may represent a marker for the preservation of mitochondrial function.
- This study provides new insight into ischemia reperfusion and possible mechanisms and treatment for the tissue injury and organ dysfunction that is associated with this process.

It has been shown that IGF-1 can protect myocardium and other tissues against apoptosis in various animal models [40–42]. IGF-1 may also improve cardiac function in diabetic patients [41–45] and rat models of myocardial infarction and reperfusion [26]. It has been shown that IGF-1 can protect myocardium by regulating changes in proapoptotic and/or antiapoptotic molecules such as Bcl-2, Bcl-X<i>l</i> and Bax. These are all related to the mitochondrial apoptotic pathway and mitochondrial energetics [26]. This may explain, in part, how IGF-1 protects myocardium even in the later phase of reperfusion injury.

In an *ex vivo* model of myocardial ischemia and reperfusion we demonstrated that IGF-1 protects against reperfusion associated injury. We found this protective effect of IGF-1 to be correlated with elevated mtDNA:nDNA relative to baseline, and this may represent a marker of preservation of mitochondrial function. This study provides new insights into ischemia/reperfusion, and suggests possible mechanisms and treatments for the tissue injury and organ dysfunction associated with this process. The eventual benefit of this to our understanding of myocardial dysfunction in sepsis awaits further study.

**Competing interests**

None declared.

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