Cardioprotective effect of insulin-like growth factor I in myocardial ischemia followed by reperfusion
(neutrophil/myocardial reperfusion injury/apoptosis)

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ABSTRACT In the present study, the cardioprotective effects of insulin-like growth factor I (IGF-I) were examined in a murine model of myocardial ischemia reperfusion (i.e., 20 min + 24 hr). IGF-I (1–10 μg per rat) administered 1 hr prior to ischemia significantly attenuated myocardial injury (i.e., creatine kinase loss) compared to vehicle (P < 0.001). In addition, cardiac myeloperoxidase activity, an index of neutrophil accumulation, in the ischemic area was significantly attenuated by IGF-I (P < 0.001). This protective effect of IGF-I was not observed with des-(1–3)-IGF-I. Immunohistochemical analysis of ischemic–reperfused myocardial tissue demonstrated markedly increased DNA fragmentation due to programmed cell death (i.e., apoptosis) compared to nonischemic myocardium. Furthermore, IGF-I significantly attenuated the incidence of myocyte apoptosis after myocardial ischemia and reperfusion. Therefore, IGF-I appears to be an effective agent for preserving ischemic myocardium from reperfusion injury and protects via two different mechanisms—inhibition of polymorphonuclear leukocyte-induced cardiac necrosis and inhibition of reperfusion-induced apoptosis of cardiac myocytes.

Although early coronary reperfusion of the ischemic myocardium is a desired therapeutic goal, evidence indicates that reperfusion itself contributes to additional myocardial cell injury (i.e., reperfusion injury) (1). This injury is preceded by endothelial dysfunction (2), a large influx of circulating neutrophils adhering onto the coronary vascular endothelium, and other key factors possibly including programmed cell death of cardiac myocytes (i.e., apoptosis) (3).

Recently, transforming growth factor β was shown to counteract the deleterious effects of tumor necrosis factor α and oxygen-derived free radicals in myocardial ischemia and reperfusion (MI/R) (4). Another growth factor, insulin-like growth factor I (IGF-I), a 70-amino acid polypeptide of ~7.5 kDa, is known to regulate cell differentiation and proliferation in a variety of cell types (5). IGF-I binds to different cell types via two specific membrane receptors (6). Recently, IGF-I was shown to ameliorate transient ischemia-induced acute renal failure in rats (7), to rescue neurons after cerebral hypoxic–ischemic injury (8), and to prevent apoptosis in differentiated cells (9). The effects of IGF-I in ischemia may be the results of direct effects on the injured cells as suggested by the ability of IGF-I to prevent apoptosis in differentiated cells (8–10). Alternatively, IGF-I may act by reducing polymorphonuclear leukocyte (PMN) infiltration during reperfusion. Interestingly, IGF-I has been shown to be an important regulator of vascular function by stimulating NO release from the cultured vascular endothelium (11, 12). Decreased release of basal NO promotes neutrophil adherence (13) to the coronary endothelial cell surface and eventually leads to neutrophil accumulation within the myocardium several hours after reperfusion (14, 15). This latter action could lead to a down-regulation of neutrophil–endothelial interaction and protect against reperfusion injury (16, 17). Moreover, IGF-I may exert cardioprotective effects via inhibition of apoptosis in myocardial cells.

Therefore, the major purposes of this study were to determine the effects of exogenous IGF-I on (i) myocardial tissue necrosis, (ii) neutrophil accumulation in the ischemic reperfused myocardium, and (iii) induction of postreperfusion apoptosis in a well-established model of murine MI (20 min)/R (24 hr).

MATERIALS AND METHODS

Experimental Protocol. Male Sprague–Dawley rats (225–250 g) were lightly anesthetized with ether prior to surgery. MI was produced by briefly exteriorizing the heart and placing a 4.0 silk knot around the left coronary artery, approximately 2–3 mm from its origin, effectively occluding the vessel. Ischemia was maintained for 20 min, at which time the slip knot was released, initiating reperfusion. Sham-operated control rats (sham MI) underwent the same surgical procedures except that the suture that was passed under the left coronary artery was not tied. After 24 hr of reperfusion, rats were anesthetized with sodium pentobarbital (35 mg/kg, i.p.), and their hearts were excised and placed in ice-cold 0.9% NaCl. The left ventricular free wall (LVFW) and interventricular septum were dissected free and homogenized in cold 0.25 M sucrose (1:18; wt/vol) containing 1 mM EDTA and 1 mM 2-mercaptoethanol with a Polytron (PCU-2) homogenizer. Homogenates were centrifuged at 36,000 × g at 4°C for 30 min. The supernatants were decanted and analyzed spectrophotometrically for creatine kinase (CK) and myeloperoxidase (MPO) activities.

Rats were randomly divided into three major groups: sham MI rats, MI/R rats receiving vehicle, and MI/R rats receiving IGF-I alone or in combination with des-(1–3)-IGF-I. Human recombinant IGF-I and des-(1–3)-IGF-I were obtained from GroPep (Adelaide, Australia). IGF-I preparations were >95% pure (by HPLC) and contained <0.1 endotoxin unit/μg. IGF-I and des-(1–3)-IGF-I were administered in doses of 1 or 10 μg per rat dissolved in 0.1 mL of 0.9% NaCl diluted with 10% rat plasma to 0.5 mL. The injections were given i.p. 1 hr before induction of MI. In other studies, a single i.p. or i.v. administration of des-(1–3)-IGF-I and IGF-I or vehicle administration was given immediately before reperfusion (i.e., 20 min postischemia).

Abbreviations: CK, creatine kinase; IGF-I, insulin-like growth factor I; MI/R, myocardial ischemia and reperfusion; MPO, myeloperoxidase; PMN, polymorphonuclear leukocyte; LVFW, left ventricular free wall.

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**CK Analysis.** Protein concentration was determined by the biuret method of Gornall et al. (18). CK activity of LVFW and septum was measured by the method of Rosalki (19). The difference in CK activity between LVFW and septum was calculated by subtracting CK_{LVFW} from CK_{septum} and expressed in international units per 100 mg of protein. The CK washout from the injured LVFW compared to the noninjured septum is a useful index of tissue injury after ischemia and reperfusion (20). All CK and protein determinations were made blindly without prior knowledge of the group of origin of each rat.

**Determination of Myocardial MPO Activity.** The myocardial activity of MPO, an enzyme occurring virtually exclusively in neutrophils, was determined spectrophotometrically by the method of Bradley et al. (21) as modified by Mullane et al. (22) in supernatants of homogenized myocardium (i.e., LVFW and septum). One unit of MPO is defined as that quantity of enzyme hydrolyzing 1 mmol of peroxide per min at 25°C. The MPO activity in the LVFW was calculated by subtracting MPO_{septum} from MPO_{LVFW} and is expressed as MPO difference. All MPO determinations were made blindly without prior knowledge of the group of origin of each rat.

**In Situ Determination of Apoptosis in Ischemic Reperfused Myocardium.** For immunohistochemical analysis, eight additional rats were subjected to sham ischemia or 20 min of ischemia followed by 24 hr of reperfusion and given either 1 μg of IGF-I or its vehicle. At the end of the 24-hr reperfusion period, the hearts were removed and perfused retrogradely with K-H buffer for 2 min. Perfusion was then switched to 4% paraformaldehyde in phosphate-buffered saline (pH 7.4; 4°C) for 5 min to fix the hearts. Full-thickness slices of the ischemic and nonischemic left ventricular wall (1 mm thick and 5 mm wide) were fixed for 1.5 hr at 4°C in 4% paraformaldehyde, dehydrated, and embedded. The ventricular slices were then dehydrated in a graded series of acetone solutions (i.e., 50%, 70%, 90%, and 100%) at 4°C and subsequently embedded in methacrylate at 4°C for 12 hr. Tissue sections were placed on Vectabond-coated slides (Vector Laboratories).

Immunohistochemical procedures for apoptosis were performed by direct immunoperoxidase detection of digoxigenin-labeled genomic DNA in thin sections (ApopTag, Oncor). Tissue sections were treated with trypsin and H_{2}O_{2}. Residues of digoxigenin nucleotide were catalytically (1 hr; 37°C) added to the 3'-OH end of DNA by terminal deoxynucleotidyltransferase. Incubation with the anti-digoxigenin antibody fragment was carried out for 30 min at room temperature. Color development was performed with a diaminobenzidine substrate/H_{2}O_{2} solution (Vector Laboratories). The percentage of immunolabeled nuclei of myocytes was counted in random tissue sections as an index of the occurrence of apoptosis. Myocytes were analyzed in at least 10 separate fields for each tissue section. The number of apoptotic myocytes (i.e., peroxidase positive in the cell nucleus) was counted for each field. The number of stained myocytes was divided by the total number of myocytes and then multiplied by 100 to determine the percentage stained myocytes [(stained myocytes)/(total myocytes) × 100].

**Statistical Analysis.** All values in the text and figures are presented as means ± SEM of n independent experiments. All data were subjected to ANOVA followed by Fisher's t test. Probabilities of 0.05 or less were considered to be statistically significant.

**RESULTS**

**Effect of IGF-I on Myocardial Injury After Reperfusion.** To evaluate the extent of ischemic injury, we measured CK activity in homogenates of nonischemic (i.e., interventricular septum) or ischemic (i.e., LVFW) myocardial tissue after 24 hr of reperfusion. Sham MI/R rats receiving 10 μg of des-(1-3)-IGF-I and 10 μg of IGF-I demonstrated only a small nonsignificant difference in CK activity between septum and LVFW (Fig. 1). Sham-operated rats receiving vehicle also showed similar CK differences compared with sham MI/R rats treated with IGF-I (data not shown). However, rats subjected to coronary occlusion and reperfusion and given only the vehicle had significant loss of CK from the left ventricular wall (P < 0.001 compared with sham MI rats) (Fig. 1). In contrast, the combination of 1 μg of IGF-I/des-(1-3)-IGF-I given i.p. 1 hr before ischemia significantly attenuated CK loss from the ischemic–reperfused myocardium. Ten micrograms of IGF-I/des-(1-3)-IGF-I exerted a significant degree of cardioprotection that was only slightly improved over that of 1 μg of IGF-I/des-(1-3)-IGF-I.

To determine which of the two different IGF-I forms (i.e., full-length IGF-I and des-(1-3)-IGF-I) was more active, we studied the effect of single administration of either 1 μg of full-length IGF-I or des-(1-3)-IGF-I and compared them to the combination of both IGF-I forms. One microgram of IGF-I significantly attenuated CK loss from the ischemic–reperfused myocardium compared to vehicle-treated rats (P < 0.01) (Fig. 2). There was no statistical difference between the CK loss observed after administration of IGF-I alone and that occurring after the combination of 1 μg of IGF-I/des-(1-3)-IGF-I. In contrast, 1 μg of des-(1-3)-IGF-I failed to prevent the significant depletion of CK from the LVFW after MI/R. There was no significant difference in CK loss in response to des-(1-3)-IGF-I compared to that of vehicle-treated rats (Fig. 2). In addition, administration of the combination of 1 μg of IGF-I and des-(1-3)-IGF-I 1 min before reperfusion (i.e., 20 min postocclusion) whether administered i.p. or i.v. exerted no significant cardioprotective effects. These results clearly indicate that (i) IGF-I prevents myocardial injury after reperfusion of the ischemic myocardium, (ii) full-length IGF-I is active, whereas des-(1-3)-IGF-I is not active, and (iii) pretreatment with IGF-I appears to be required for cardioprotection.

**Neutrophil Accumulation in the Ischemic–Reperfused Myocardium.** Accumulation of neutrophils in the ischemic region during reperfusion has been thought to be a major mechanism responsible for reperfusion injury. We therefore measured MPO activity in the nonischemic septum and the ischemic LVFW portions of the myocardium and used the difference between the two values as an index of neutrophil accumulation. Sham-operated control rats receiving IGF-I showed small MPO differences similar to those obtained in sham MI/R rats given vehicle (data not shown). In contrast, MI/R rats receiving only the vehicle exhibited a marked increase in MPO activity in the LVFW, indicating an increased neutrophil...
accumulation in the reperfused myocardium after ischemia. Fig. 3 summarizes these data. However, i.p. administration of 1 and 10 μg of IGF-I/des-(1-3)-IGF-I 1 hr before ischemia significantly attenuated the increase in MPO activity in the ischemic–reperfused myocardium. One microgram of IGF-I/des-(1-3)-IGF-I exerted a partial inhibition of PMN accumulation, which was further increased by 10 μg of IGF-I/des-(1-3)-IGF-I. We also studied the effects of single administration of the two different forms of IGF-I. One microgram of IGF-I significantly attenuated the MPO increase in the ischemic–reperfused myocardium compared with vehicle-treated rats (P < 0.01) (Fig. 4). We did not observe any statistical difference between the effects of administration of IGF-I alone and the combination of 1 μg of IGF-I/des-(1-3)-IGF-I. However, 1 μg of des-(1-3)-IGF-I failed to prevent the increased MPO activity in the LVFW, and there was no significant difference in MPO activity compared to vehicle-treated rats (Fig. 4). In addition, administration of the combination of 1 μg of IGF-I and des-(1-3)-IGF-I 1 min before reperfusion either i.p. or i.v. exerted no inhibition of neutrophil accumulation in the reperfused myocardium. These results indicate that the cardioprotective effect of IGF-I may be partially related to inhibition of neutrophil accumulation in the ischemic–reperfused myocardium.

![Diagram](https://example.com/diagram1.png)

**FIG. 2.** Effect of single administration of full-length IGF-I and des-(1-3)-IGF-I on myocardial injury after reperfusion of ischemic myocardium. Differences in myocardial CK activity in the LVFW and septum are expressed in international units per 100 mg of tissue. Rats were treated with 1 μg of IGF-I, des-(1-3)-IGF-I, or a combination of both. Values are means ± SEM for 6–10 rats in each group. NS, not significant.

**FIG. 3.** MPO activity expressed as difference in LVFW and septum MPO activity expressed as units per 100 mg of tissue (wet weight) for sham MI rats and MI/R rats treated with 1 and 10 μg of IGF-I and des-(1-3)-IGF-I and vehicle. Heights of bars are means; brackets represent ±SEM for 6–8 samples in each group.

**FIG. 4.** Effect of single administration of full-length IGF-I and des-(1-3)-IGF-I on neutrophil accumulation after reperfusion of ischemic myocardium. Difference of myocardial MPO activity in the LVFW and septum is expressed as MPO units per 100 mg of tissue (wet weight). Rats were treated with 1 μg of IGF-I, des-(1-3)-IGF-I, or a combination of both. Values are means ± SEM for 6–10 rats in each group. NS, not significant.

**Immunohistochemical Localization of Apoptosis After MI/R.** The presence of apoptosis in the ischemic–reperfused myocardium was determined by direct immunoperoxidase detection of digoxigenin-labeled genomic DNA in thin sections of the myocardium. Nonischemic sections of heart tissue as well as sections from sham MI hearts failed to demonstrate significant immunostaining (i.e., <5% of the myocytes were stained) (Fig. 5A and 6). Similarly, immunohistochemical preparations, in which either the digoxigenin–dUTP or the antibody–peroxidase conjugate was replaced with nonimmune serum, failed to exhibit any labeling of myocardial or other cell types. In contrast to these controls, apoptosis was clearly evident in ischemic–reperfused cardiac sections obtained from untreated ischemic–reperfused hearts (i.e., 62% ± 5% myocytes; P < 0.01 compared to sham MI rats) (Figs. 5B and 6). Since DNA degradation also occurs nonspecifically in necrotic myocardium, we evaluated apoptosis only in areas that did not demonstrate typical signs of necrosis (i.e., loss of membrane integrity, cell lysis, or swelling). Intense immunostaining was evident in myocytes as well as in infiltrating leukocytes. Ischemic–reperfused myocardial tissue of rats treated with 1 μg of IGF-I demonstrated remarkably diminished immunostaining (i.e., 28% ± 3% myocytes, P < 0.01 compared to rats receiving vehicle only), indicating reduced occurrence of apoptosis after IGF-I treatment (Figs. 5C and 6). These results indicate that reperfusion of the ischemic myocardium results in induction of apoptosis in cardiac tissue and that IGF-I treatment appears to be an effective inhibitor of apoptosis.

**DISCUSSION**

Our results clearly show that IGF-I, when administered 1 hr prior to ischemia at 1–10 μg, markedly retards postreperfusion cardiac necrosis (P < 0.01). IGF-I (i.e., 1–10 μg per rat) was injected i.p. to allow a gradual absorption with prolonged effects rather than a rapid pulsed onset. The circulating half-life of IGF-I is reported to be on the order of 3–8 hr (5). Since IGF-I binds to at least six different binding proteins that are necessary for its biologic action (6), probably only small changes in free IGF-I levels occurred in our experiments. Binding of IGF-I to its receptors leads to autophosphorylation of the β subunit of the receptor and to subsequent activation of a tyrosine kinase (6). The cardioprotective effects of IGF-I are not likely attributed to increases in glucose utilization, since IGF-I does not cross-react with the insulin receptor and because IGF-I is only 1–10% as potent as insulin (5).
FIG. 6. Percentage of nuclei staining positive for peroxidase substrate (i.e., apoptotic cell nuclei) in sham-operated MI rat, MI/R rat treated with vehicle, and MI/R rat treated with 1 μg of full-length IGF-I. Values are means ± SEM for 4–6 histologic sections in each group.

derived NO to inhibit platelet aggregation (25), to prevent neutrophil adherence to the endothelium (14), and to quench superoxide radicals at concentrations below vasodilator concentrations (15, 26). Thus, increased release of NO at or near the endothelial surface promotes a variety of antiinflammatory and antifibrotic effects that could be important in attenuating reperfusion injury.

One important component of the myocardial salvage afforded by IGF-I is likely caused by its ability to diminish neutrophil accumulation in the ischemic–reperfused myocardium. Neutrophils are known to be involved in murine MI/R injury, and we observed significant increases in MPO activity of untreated ischemic myocardial tissue. Adhered and activated neutrophils release a variety of cytotoxic mediators (e.g., H₂O₂, superoxide anion, hydroxyl radical, and elastase), which can lead to coronary endothelial dysfunction and myocardial injury (4, 27–29). In contrast, IGF-I treatment resulted in significantly lower MPO activities in the reperfused myocardium.

The adhesion process starts with neutrophil rolling, continues with tight adhesion, and can lead to transmigration of neutrophils into the extravascular space (30–32). It is unclear whether IGF-I directly interacts with a specific adhesion molecule located either on leukocytes or on the vascular endothelium. However, in other studies, NO has been shown to be an important regulator of P-selectin and ICAM-1 expression, since inhibition of endogenous NO resulted in increased expression of P-selectin (33) and exogenous NO inhibited expression of ICAM-1 (34). Thus, there might be additional mechanisms for IGF-I-mediated cardioprotection. These effects could be explained by inhibition of PMN–endothelium interaction and subsequent reduced release of PMN mediators, leading to lesser myocardial tissue injury. This antineutrophil property of IGF-I appears to be a key mechanism of its amelioration of reperfusion injury.

Recently, Gottlieb et al. (3) have demonstrated that MI/R on the rabbit heart induces apoptosis in the reperfused myocardial cells and that this might be important for delayed myocyte cell death. Apoptosis was found to occur only after reperfusion of the ischemic myocardium and thus appears to be a contributory mechanism to reperfusion injury (3). Our results demonstrating end-labeling of DNA in cardiac myocyte nuclei confirm and extend these findings in a murine model of MI/R. Apoptosis represents a control mechanism in morphogenesis and cell turnover of cells in adult tissues. Apoptosis can be morphologically characterized by cell shrinking, loss of cell contacts, and aggregation of chromatin (35, 36). Membrane-bound apoptotic bodies are formed that contain intact cell organelles and condensed chromatin (35, 36). These bodies are
phagocytosed by macrophages or neighboring cells. Apoptosis can be triggered by cytokines such as tumor necrosis factor α (35), which can also contribute to reperfusion injury. Recently, IGF-I has been shown to inhibit apoptosis, resulting in improved cell survival within the nervous system (8, 9). In our study, IGF-I reduced the incidence of apoptosis in the ischemic–reperfused myocardium. The results of Gottlieb et al. (3) clearly show that reperfusion-induced apoptosis is independent of neutrophil accumulation in the reperfused myocardium. Therefore, the antiapoptotic actions of IGF-I in the present experiments are presumably independent of its effects on PMN sequestration.

In conclusion, we have demonstrated that in vivo administration of IGF-I attenuates both myocardial necrosis and apoptosis of cardiomyocytes resulting from MI/R. These protective effects could be at least partially attributed to reduced PMN accumulation after IGF-I administration in the reperfused myocardium, subsequently reducing cardiac necrosis. Furthermore, these in vivo results highlight the important role of growth factors as potentially useful agents in alleviating necrosis and apoptosis in inflammatory states such as that occurring after reperfusion of the ischemic myocardium.

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