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Relaxin Protects Against Myocardial Injury Caused by Ischemia and Reperfusion in Rat Heart

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Myocardial injury caused by ischemia and reperfusion comes from multiple pathogenic events, including endothelial damage, neutrophil extravasation into tissue, platelet and mast cell activation, and peroxidation of cell membrane lipids, which are followed by myocardial cell alterations resulting eventually in cell necrosis. The current study was designed to test the possible cardioprotective effect of the hormone relaxin, which has been found to cause coronary vessel dilation and to inhibit platelet and mast cell activation. Ischemia (for 30 minutes) was induced in rat hearts *in vivo* by ligature of the left anterior descending coronary artery; reperfusion (for 60 minutes or less if the rats died before this predetermined time) was induced by removal of the ligature. Relaxin (100 ng) was given intravenously 30 minutes before ischemia. The results obtained showed that relaxin strongly reduces 1) the extension of the myocardial areas affected by ischemia-reperfusion-induced damage, 2) ventricular arrhythmias, 3) mortality, 4) myocardial neutrophil number, 5) myeloperoxidase activity, a marker of neutrophil accumulation, 6) production of malonyldialdehyde, an end product of lipid peroxidation, 7) mast cell granule release, 8) calcium overload, and 9) morphological signs of myocardial cell injury. This study shows that relaxin can be regarded as an agent with a marked cardioprotective action against ischemia-reperfusion-induced myocardial injury. *(Am J Pathol 1998, 152:1367–1376)*

Cardiac ischemia results in endothelial injury followed by myocardial dysfunction and damage. Paradoxically, reperfusion of ischemic heart, although providing the cells with oxygen and trophic substances, further exacerbates tissue damage because it promotes accumulation of neutrophils and formation of oxygen-derived free radicals.[1–6] In particular, endothelial injury caused by hypoxia enhances neutrophil adherence to the coronary endothelium[7] as well as platelet adhesion and aggregation.[8] Accumulation of neutrophils in the myocardium begins during ischemia and rapidly accelerates during reperfusion.[6,9] This leads to the production of large amounts of oxygen-derived free radicals, thus extending endothelial and myocardial cell damage.[4,10–12] In turn, activation of platelets amplifies endothelial damage and favors neutrophil extravasation through the release of chemotactic factors.[8] Resident mast cells also contribute to the dysfunction of the ischemic-reperfused heart through the release of mediators, such as histamine,[13,14] which favors neutrophil adhesion to the endothelium[15] and contributes to the development of arrhythmias.[16,17]

Our previous studies showed that the peptide hormone relaxin (RLX) is a powerful dilator of blood vessels in several target organs.[18] In particular, RLX markedly enhances the coronary flow in rat and guinea pig hearts.[19] Moreover, RLX depresses platelet activation and aggregation,[20] and inhibits mast cell degranulation and histamine release.[21] On these grounds, we hypothesize that RLX may have a protective action against ischemia-reperfusion-derived myocardial injury. The current study was designed to test this hypothesis using an *in vivo* model of ischemia-reperfusion in the rat heart.

Materials and Methods

Animals

Forty-eight male albino rats, Wistar strain, weighing 250 to 300 g (Morini, Reggio Emilia, Italy) were used. They were quarantined for 7 days at 22 to 24°C with a 12-hour light/12-hour dark cycle before use. Standard laboratory chow (Rodentia, Bergamo, Italy) and water were available *ad libitum*. The experimental protocol was designed in compliance with the recommendations of the European Economic Community (86/609/CEE) for the care and use of laboratory animals and was approved by the animal care committee of the University of Florence (Italy). The rats were randomly distributed in six groups: three

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groups were composed of 10 animals each and three groups were composed of 6 animals each.

Treatments

The rats were anesthetized by intraperitoneal injection of ketamine (Parke Davis, Milan, Italy; 150 mg/kg body weight). A cannula was inserted into the trachea and the animals were ventilated with air using a Palmer pump (U. Basile, Comerio, Italy). Subcutaneous peripheral limb electrodes were inserted and electrocardiogram (ECG) was continuously recorded for the entire duration of the experiment. All rats underwent thoracotomy at the fifth left intercostal space, the pericardium was opened and a loose 00 braided silk suture was placed around the left anterior descending coronary artery approximately 1 to 2 mm below its origin. To facilitate the successive removal of the suture, a small silicon ring was inserted in the silk thread below the knot. Then, the chest was closed by a silk suture to minimize heart displacement, taking care to leave the ends of the coronary suture threads emerging from the surgical wound. Rats were allowed to equilibrate for 20 minutes to enable ECG values to stabilize. Ischemia was induced by tightening the threads of the coronary suture and was maintained for 30 minutes. Reperfusion was obtained by reopening the chest and cutting the ligature around the coronary artery. The duration of reperfusion was predetermined to 60 minutes. In the animals that did not survive the entire reperfusion period, reperfusion lasted until cessation of the cardiac activity as revealed in ECG recordings. In all of the animals, survival time was registered. To exclude that premature mortality of rats be caused by the surgical procedures or individual abnormalities, rats showing ECG signs of impaired cardiac function during the stabilization period before induction of ischemia or soon after the coronary artery ligature were excluded from the experiments.

Group 1

This group consisted of RLX-treated rats undergoing ischemia and reperfusion (n = 10). These rats were treated with highly purified porcine RLX (2500 to 3000 U/mg), prepared according to Sherwood and O'Byrne.[22] RLX was kindly provided by Dr. O. D. Sherwood (University of Illinois at Urbana Champaign, Urbana, IL). The hormone was dissolved in saline at a concentration of 200 ng/ml, and 500 μl of the solution were injected into the penile vein 30 minutes before induction of ischemia. This RLX dose was calculated to provide a blood concentration corresponding to the RLX concentration that was shown to be effective in increasing coronary flow in isolated rat hearts.[19] The half-life of RLX has been evaluated to be approximately 2 hours.[23] This fact allows for the peptide to be available to the heart for the overall experimental period. The rats of this group were used for biochemical and morphological analyses.

Group 2

This group consisted of rats undergoing ischemia and reperfusion (n = 10). These rats were treated with 500 μl of the vehicle alone, ie, saline, injected into the penile vein 30 minutes before induction of ischemia. They were used for biochemical and morphological analyses.

Group 3

This group consisted of sham-operated rats (n = 10). These rats were injected with 500 μl of saline and then underwent the same surgical procedures as above but without the tightening of the coronary suture. They were used as controls for biochemical and morphological analyses.

Group 4

This group consisted of RLX-treated rats undergoing ischemia and reperfusion (n = 6). These rats were treated similarly to those of group 1. They were used for assessment of the extension of the left ventricular myocardium undergoing ischemic damage as described below.

Group 5

This group consisted of rats undergoing ischemia and reperfusion (n = 6). These rats were treated similarly to those of group 2. They were used for assessment of the extension of the left ventricular myocardium undergoing ischemic damage.

Group 6

This group consisted of sham-operated rats (n = 6). These rats were treated similarly to those of group 3. They were used as controls for the groups used for assessment of the extension of the left ventricular myocardium undergoing ischemic damage.

At the end of reperfusion, or at the moment of cessation of the cardiac activity in the rats that did not survive the predetermined reperfusion period, the chest was reopened and the hearts quickly removed. In the animals from groups 1 to 3, the anterior left ventricular wall, 3-mm distal to the ligature to exclude tissue areas damaged by the surgical procedure, was excised. This was cut into fragments, some of which, chosen at random, were used for morphological studies and the others for biochemical assays. In the animals from groups 4 and 5, the extension of the left ventricular myocardium undergoing damage caused by ischemia-reperfusion was determined by the nitro blue tetrazolium dye exclusion method.[24] The sham-operated hearts from the rats of group 6 were treated with the same method and were used as negative controls. On removal, the hearts were attached to a Langendorff's apparatus through a cannula introduced into the aorta and perfused with 10 ml of 1% nitro blue tetrazolium

(Sigma, St. Louis, MO) dissolved in a modified Tyrode solution,[25] pH 7.4, at a constant pressure of 40 cm of water and at 37°C. In this way, the aortic semilunar valve remains closed and the dye enters directly into the coronary arteries. The hearts were maintained at 37°C for 20 minutes. Following this treatment, the normal myocardium shows an intense blue staining reaction because of the presence of dehydrogenase enzymes, whereas ischemia-reperfusion-injured regions remain unstained. Thus, the latter regions appear as clearly delineated, unstained zones. The hearts were detached from the cannula, weighed, fixed in buffered 4% formaldehyde for 12 hours, and the ventricles sectioned in 2-mm transverse slices from the apex to the ligature. In each slice, the boundaries of the unstained area on the upside surface were traced onto a superimposed acetate sheet and the encircled area was measured by computer-assisted morphometry, as described below. In each slice, the volume of the damaged myocardium was calculated by multiplying the unstained surface area for the thickness of the slice (assuming that the unstained area was similar on the upside and downside surfaces). In each heart, the total volume of the damaged myocardium was calculated as the sum of the partial values of the different slices. To allow a comparison of the extension of myocardial injury between hearts of different size, the total volume of the damaged myocardium was divided by the heart weight (grams).

Analysis of ECG

The development of disturbances of the cardiac rhythm, such as ventricular tachycardia (VT) and ventricular fibrillation (VF), which are known to be associated with myocardial ischemia and reperfusion,[26] has been evaluated in ECG recordings of all of the rats subjected to study during both coronary artery occlusion and reperfusion. According to Gelvan et al,[27] VT was recognized as three or more consecutive premature ventricular contractions, and VF was recognized as irregular modulating baseline. A heart was considered to be in normal sinus rhythm when normal sinus complexes occurring at a regular rate were observed. The values are reported as the number of rats with heart arrhythmias over the total number of animals of each group. ECG analysis, which allows to identify the moment of the cessation of cardiac activity, was also used to evaluate the survival period of the rats during the postischemic reperfusion phase.

Evaluation of Myeloperoxidase Activity

Myeloperoxidase (MPO) activity can be used as a marker for neutrophil accumulation in tissues.[28,29] MPO activity was evaluated according to Bradley et al.[28] Briefly, frozen samples of left ventricular tissue weighing approximately 100 mg were homogenized in 1.5 ml of 50 mmol/L potassium phosphate buffer, pH 6. One milliliter of the homogenate was centrifuged at 10,000 × *g* for 10 minutes, and the pellet was suspended in 1 ml of potassium phosphate buffer (50 mmol/L), pH 6, containing 0.5% hexadecyltrimethylammonium bromide (Sigma) to negate the pseudoperoxidase activity of hemoglobin and to solubilize membrane-bound MPO. The suspensions were treated with three cycles of freezing-thawing, sonicated on ice for 10 seconds, and centrifuged at 12,000 × *g* for 10 minutes. MPO activity was determined in the supernatants. Briefly, 0.1 ml of the supernatant was mixed with 2.9 ml of potassium phosphate buffer (50 mmol/L), pH 6, containing 0.19 mg/ml of *o*-dianisidine chloride and 0.0005% H_2O_2 as a substrate for MPO.[30] Oxidized *o*-dianisidine forms a stable chromophore absorbing at a 460-nm wave length. The absorbance was determined spectrophotometrically over 2 minutes. The values of tissue MPO activity were obtained by comparison with standard concentrations of *o*-dianisidine in the presence of excess H_2O_2. One unit of MPO activity is defined as that required to degrade 1 μmol of H_2O_2 per minutes at 25°C. Protein concentration was determined with the Bradford method.[31] The results are expressed as mU/mg of proteins.

Determination of Malonyldialdehyde

Malonyldialdehyde (MDA) is an end-product of peroxidation of cell membrane lipids caused by oxygen-derived free radicals and is considered a reliable marker of myocardial cell damage.[32] It was determined by measurement of the chromogen obtained from the reaction of MDA with 2-thiobarbituric acid according to Aruoma et al.[33] Approximately 100 mg of myocardial tissue were homogenized with 1 ml of 50 mmol/L Tris-HCl buffer containing 180 mmol/L KCl and 10 mmol/L EDTA, final pH 7.4, using a tissue homogenizer (Ing. Terzano, Milan, Italy). 0.5 ml of 2-thiobarbituric acid (1% w/v) in 0.05 mol/L NaOH and 0.5 ml of HCl (25% w/v in water) were added to 0.5 ml of sample. The mixture was placed in test tubes, sealed with screw caps, and heated in boiling water for 10 minutes. After cooling, the chromogen was extracted in 3 ml of 1-butanol, and the organic phase was separated by centrifugation at 2000 × *g* for 10 minutes. The absorbance of the organic phase was read spectrophotometrically at a 532-nm wave length. Protein concentration was determined according to Bradford.[31] The values are expressed as nanomoles of thiobarbituric acid-reactive substances (MDA equivalents)/mg of protein, using a standard curve of 1,1,3,3-tetramethoxypropane.

Evaluation of Calcium Content

Excessive calcium influx is a critical event accompanying irreversible injury in myocardial ischemia-reperfusion.[34,35] The calcium content of the myocardial tissue was measured by atomic absorption spectrometry.[36] Briefly, the tissue fragments weighing approximately 30 g were rinsed thoroughly in calcium-free-buffered solution, dried in an oven at 80°C, and digested overnight with 65% HNO_3 (100 μl/10 mg of dry tissue). After addition of 32% HCl (150 μl/10 mg of dry tissue) the samples were dried at 45°C under nitrogen. At the moment of the assay the samples were suspended in 50 μl of 32% HCl and

Table 1. Scoring Method of Myocyte and Endothelial Injury

Injury	Score	Degree of injury	Description
Myocyte	0	Normal myocyte	
	1	Slight	Mild intracellular edema
			Mild mitochondrial swelling
	2	Moderate	Mild intracellular edema
			Contracture of myofibrils
			Marked mitochondrial swelling with clearing of matrix
			Occasional focal clumping of mitochondrial cristae
			Mild nuclear chromatin clumping
	3	Severe	Severe mitochondrial swelling with loss of cristae
			Presence of intramitochondrial dense granules
			Disarrangement of myofibrils
			Plasma membrane rupture
			Nuclear degeneration (apoptosis or karyolysis)
Endothelial	0	Normal endothelium	
	1	Slight	Mild to moderate endothelial swelling
	2	Moderate	Marked endothelial swelling
			Decreased pinocytotic vesicles
			Mitochondrial swelling
	3	Severe	Severe mitochondrial swelling with loss of cristae
			Plasma membrane rupture
			Nuclear degeneration (apoptosis or karyolysis)
			Neutrophil adhesion and extravasation

added with lanthanum chloride ($LaCl_3 \cdot 7H_2O$) to provide a final concentration of 1% lanthanum (w/v). The amounts of calcium in the samples were read in an atomic absorption spectrophotometer (Perkin–Elmer 303, Überlingen, Germany) at a 422-nm wave length. The relevant values were determined by comparison with a standard curve obtained with increasing concentrations of $CaCl_2$ and expressed as ng of calcium/mg of tissue (dry weight).

Morphology

Five randomly chosen small tissue fragments, taken from the hearts of the rats of groups 1 to 3, were fixed in cold 4% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.4, for 3 hours at room temperature and were postfixed in 1% osmium tetroxide in 0.1 mol/L phosphate buffer, pH 7.4, for 1 hour at 4°C. They were then dehydrated in graded acetone, passed through propyleneoxide and embedded in Epon 812 (Fluka, Buchs, Switzerland). Light microscopic examination was carried out on 2-μm-thick semithin sections and stained with toluidine blue-sodium tetraborate. In each fragment, at least two different semithin sections cut at different levels were examined. Electron microscopic examination was carried out on ultrathin sections stained with uranyl acetate and alkaline bismuth subnitrate and viewed under a Siemens Elmiskop 102 electron microscope at 80 kV. In each fragment, two series of six to eight ultrathin sections cut at two different levels (each series put on an electron microscopy grid) were examined and photographed. Myocyte and microvascular endothelium injury were quantitated from electron micrographs (final magnifications ranging from ×3000 to ×20,000) using a method used previously for similar purposes[37] with minor modifications. The criteria used are reported in Table 1. Each animal was assigned a separate score for myocyte and endothelial injury. The average values (mean ± SE) of each group were then calculated.

Two randomly chosen tissue fragments, taken from the hearts of the rats of groups 1 to 3, were fixed by immersion in isotonic formaldehyde acetic acid, dehydrated in graded ethanol, and embedded in paraffin wax. Sections, 5 μm thick, were cut and stained with astra blue, which selectively binds to heparin contained in mast cell granules.[38] In each fragment, at least two sections cut at different levels were examined. These sections underwent analysis by computer-assisted morphometry to evaluate the light transmittance across mast cells, which is inversely related to their content in secretory granules. Methodological details are described below.

Computer-Assisted Morphometry

The surface areas of the ischemia-reperfusion-injured myocardium on slices of hearts stained with nitro blue tetrazolium (groups 4 to 6) were measured on the corresponding profiles reported on the acetate sheets. These profiles were registered through a CCTV television camera (Sony, Tokyo, Japan) interfaced with an Apple Macintosh LC III personal computer through a Videospigot card (Supermac, Sunnyvale, CA). The program that was used (1.49 Image Analysis Program, National Institutes of Health, Bethesda, MD) enables the areas encircled by each profile to be measured.

Evaluation of light transmittance across mast cells was performed according to the method described previously for similar purposes.[21] The mast cells were viewed by a CCTV television camera (Sony) applied to a Reichert-Jung Microstar IV light microscope (Cambridge Instruments Inc., Buffalo, NY) with a ×100 oil immersion objective and interfaced with an Apple Macintosh LC III personal computer through a Videospigot card (Super-

mac). The card allows for the light transmitted across the microscopic slide to be determined within a range of 256 gray levels, which are comprised between 0 (black level) and 255 (white level). The card also allows for a digitized image of mast cells to be reproduced on the basis of the values estimated. Measurements of transmittance were carried out using the same NIH 1.49 image analysis program cited above. In each experimental group, the transmittance of 100 different mast cells, 10 from each animal of the group (five per fragment), was analyzed and the mean transmittance value (mean ± SE) was then calculated.

Statistical Analysis

The reported data are expressed as mean ± SE. For ECG analysis, significance of differences between the experimental groups was assayed by the X^2 test. In the biochemical and morphometric assays, in the evaluation of survival of the rats during reperfusion, and in the quantitation of myocyte and endothelial injury by electron microscopy, the distribution of the measured values in the different experimental groups was assessed to be gaussian. Statistical analysis was performed by either one-way analysis of variance (ANOVA) test followed by Student-Newman-Keuls multiple comparison test or by Student's *t*-test for unpaired values. Calculations were carried out using a GraphPad Prism 2.0 statistical program (GraphPad Software, San Diego, CA). $P < 0.05$ was considered significant.

Results

Extension of Left Ventricular Myocardium with Ischemia-Reperfusion-Induced Injury

The results of computer-assisted morphometry on the ischemic and reperfused hearts stained with nitro blue tetrazolium showed that, compared with the rats not treated with RLX, in the RLX-treated rats the extension of the damaged myocardium is significantly reduced to nearly a half. In four of six sham-operated hearts, no signs of myocardial damage were appreciable; in the remaining two, a very small portion of damaged myocardium was observed in the ventricular slices closest to the untied thread (Figure 1).

ECG Analysis and Duration of Survival during the Postischemic Reperfusion Phase

Examination of ECG recordings from the rats subjected to myocardial ischemia and reperfusion showed that RLX reduced the occurrence of ventricular arrhythmias. During ischemia, VT and VF occurred in 7 of the 16 animals not treated with RLX, whereas ventricular arrhythmias were found in none of the 16 RLX-treated rats ($P < 0.0003$). During reperfusion, VT and VF occurred in 13 of the 16 rats not treated with RLX, whereas these arrhythmias were present in 4 of the 16 RLX-treated rats ($P <$

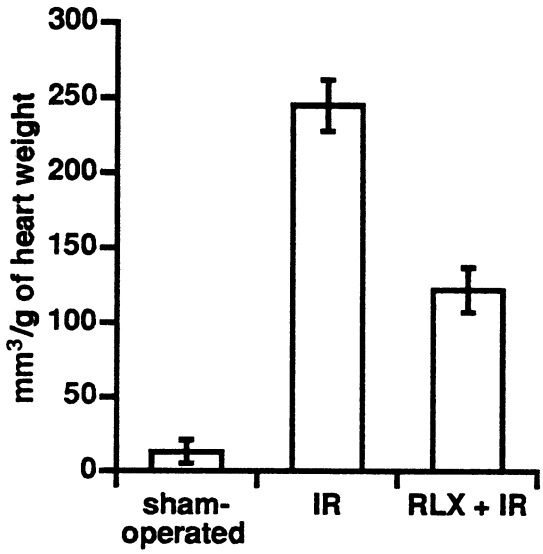

Figure 1. Extension of left ventricular myocardium with ischemia-reperfusion-induced injury as evaluated by computer-assisted morphometry on hearts stained with nitro blue tetrazolium. Compared with the rats not treated with RLX from group 5 (IR), in the RLX-treated rats from group 4 (RLX + IR) the extension of the damaged myocardium is significantly reduced. The sham-operated rats from group 6 show minimal areas of damaged myocardium. Significance of differences between groups (one-way ANOVA; each group $N = 6$): $P < 0.001$.

0.001). In the sham-operated rats, VT was only observed in 1 of the 16 animals of this group starting 60 minutes from placement of the silk thread around the left coronary artery.

During ischemia, none of the animals died. During postischemic reperfusion, some animals died in both the RLX-treated and untreated groups. However, the number of animals that survived was higher and the duration of survival was longer in the RLX-treated group than in the untreated one (Figure 2). All of the sham-operated rats survived the entire experimental period.

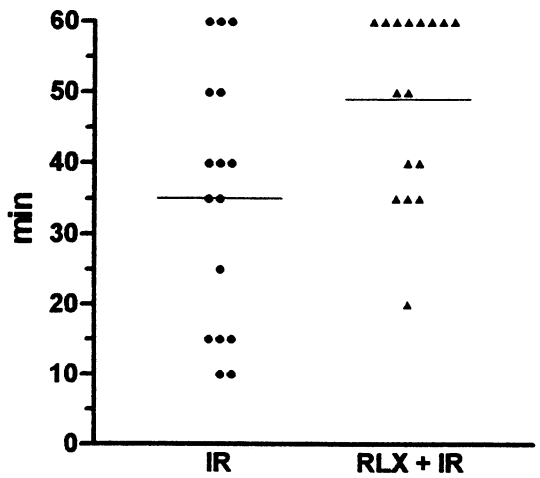

Figure 2. Survival plot for rats during the postischemic reperfusion period. The mean survival time increased significantly in the RLX-treated rats (RLX + IR) from groups 1 and 4 ($N = 16$) compared with the untreated ones (IR) from groups 2 and 5 ($N = 16$). Significance of differences (Student's *t*-test): $P < 0.02$.

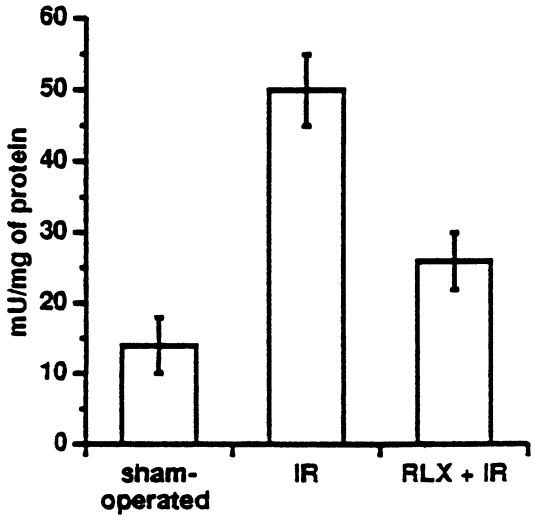

Figure 3. MPO activity in myocardial tissue. Compared with the sham-operated hearts (group 3, $N = 10$), the ischemic-reperfused hearts of the rats not given RLX (IR, group 2, $N = 10$) show a significant increase in MPO activity. This effect is blunted by RLX treatment (RLX + IR, group 1, $N = 10$). Values are mean ± SE. Significance of differences (one-way ANOVA): IR *versus* sham-operated and RLX + IR, $P < 0.001$; sham-operated *versus* RLX + IR, $P < 0.05$.

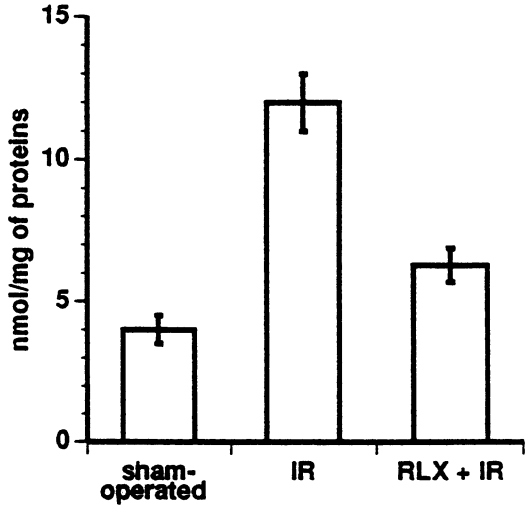

Figure 4. MDA production in myocardial tissue. Compared with the sham-operated hearts (group 3, $N = 10$), the ischemic-reperfused hearts of the rats not given RLX (IR, group 2, $N = 10$) show a significant increase in MDA. This effect is blunted by RLX treatment (RLX + IR, group 1, $N = 10$). Values are mean ± SE. Significance of differences (one-way ANOVA): IR *versus* sham-operated, $P < 0.001$; IR *versus* RLX + IR, $P < 0.001$; sham-operated *versus* RLX + IR, $P < 0.05$.

Biochemical Studies

The results of the biochemical assays for the markers of ischemia-reperfusion-induced myocardial injury showed that, in the sham-operated hearts used as controls, MPO activity, MDA production, and calcium content attained very low levels. In the ischemic-reperfused hearts, MPO activity, MDA production, and calcium content were significantly elevated. On the contrary, these parameters were significantly reduced to nearly a half in the ischemic-reperfused hearts from the RLX-treated rats as compared with the ischemic-reperfused hearts of the rats that did not receive RLX (Figures 3, 4, and 5).

Morphological Studies

Substantial differences were observed between the myocardial tissue taken from the rats of groups 1 to 3.

Light microscopic examination of semithin sections from the sham-operated hearts showed that the myocardium had a normal appearance. In particular, neutrophils adherent to the coronary endothelium or in the intramuscular connective tissue septa were not observed, nor were intercellular edema and blood vessel dilation (Figure 6A). In the ischemic-reperfused hearts, numerous neutrophils were seen adherent to the endothelium of microvessels or migrated into the perivascular connective tissue (Figure 6B). Intercellular edema was also seen. In contrast, in the ischemic-reperfused hearts from the RLX-treated rats, neutrophil adhesion and extravasation was an occasional finding. Microvessels were markedly dilated (Figure 6C).

Electron microscopic examination of specimens from the sham-operated rats showed that the coronary microvessels as well as the myocardial cells had normal features (Figure 7). In the myocardium from the ischemic-reperfused hearts, numerous neutrophils were seen passing across the microvascular lining through gaps between endothelial cells. The endothelial cells often showed cytoplasmic swelling, reduction of pinocytotic vesicles, and mitochondrial swelling. Myocardial cell alterations from moderate to severe were also seen. They consisted in intracellular edema, mitochondrial swelling, presence of numerous intramitochondrial dense granules consistent with cation deposits (Figure 8A), hypercontraction of myofibrils, and loss of the characteristic cross banding. Images of cell necrosis, including disarrange-

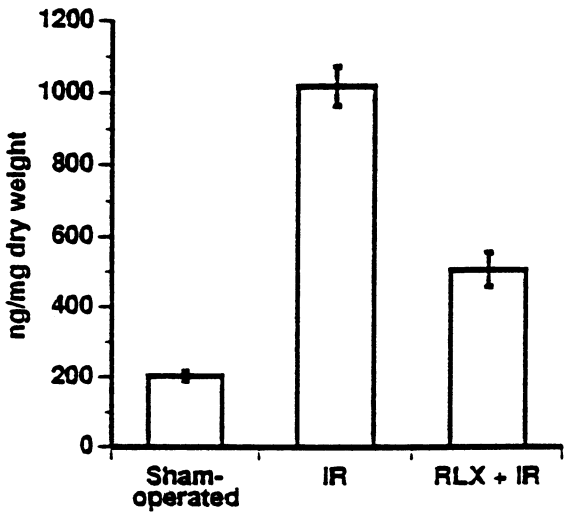

Figure 5. Calcium content in myocardial tissue. Compared with the sham-operated hearts (group 3, $N = 10$), the ischemic-reperfused hearts of the rats not given RLX (IR, group 2, $N = 10$) show a significant increase in calcium content. This effect is blunted by RLX treatment (RLX + IR, group 1, $N = 10$). Values are mean ± SE. Significance of differences (one-way ANOVA): IR *versus* sham-operated, $P < 0.001$; IR *versus* RLX + IR, $P < 0.001$; sham-operated *versus* RLX + IR, $P < 0.001$.

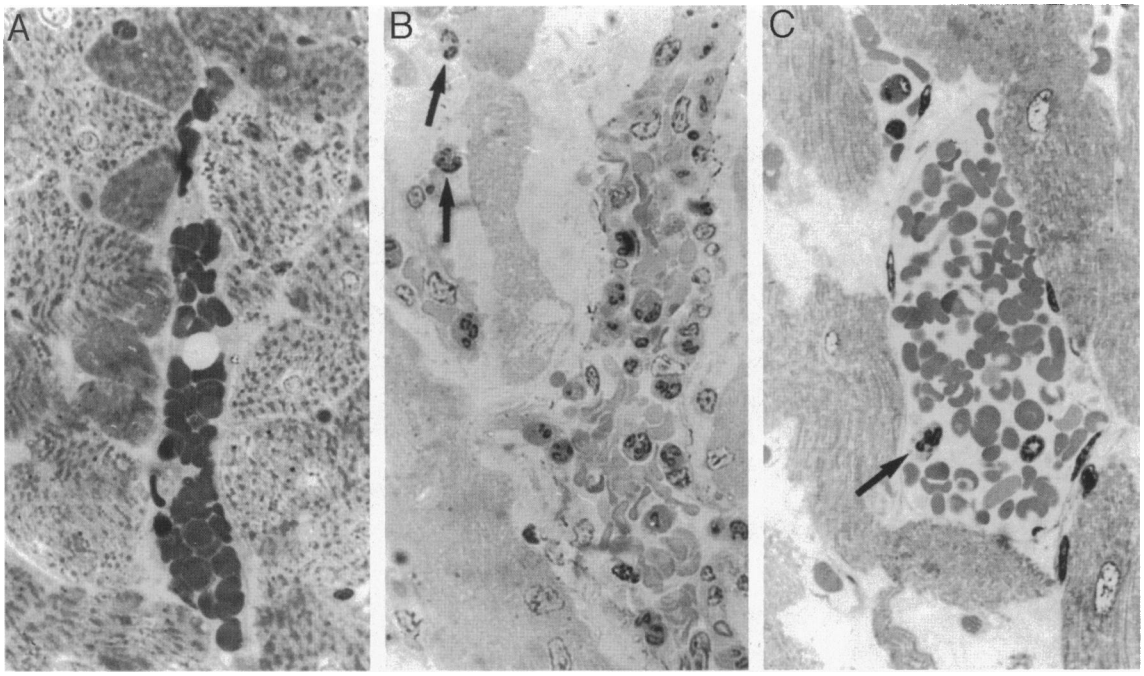

Figure 6. A: Sham-operated heart. A venule is seen with no neutrophils inside. Neutrophils are absent in the connective tissue. No appreciable interstitial edema can be seen. **B:** Ischemic-reperfused heart. A venule is shown with numerous neutrophils within the lumen and along the endothelium. Neutrophils can also be seen in the edematous connective tissue interposed between the myocardial cells (**arrows**). **C:** Ischemic-reperfused heart following RLX treatment. A venule is seen with a single neutrophil within the lumen (**arrow**). Neutrophils are absent at either the vascular wall and in the interstitial connective tissue. A slight intercellular edema can be seen. Light microscopy, semithin sections stained with toluidine blue-sodium tetraborate. Magnification, ×800.

ment of myofibrils (Figure 8B), detachment of myofilaments from the cell membrane at the intercalated disks, and nuclear degeneration, were also observed. At the opposite, in the ischemic-reperfused hearts from RLX-treated rats, the endothelial alterations were strongly reduced or, more often, were absent. Myocardial cells mostly had a nearly normal ultrastructure (Figure 9A). Only a minority of them did show moderate mitochondrial swelling but with very few or no intramitochondrial dense granules (Figure 9B). Quantitation of ultrastructural tissue injury revealed that endothelial cell damage is significantly increased in the ischemic-reperfused hearts as compared with the sham-operated ones (ischemic-reperfused, 2.9 ± 0.1; sham-operated, 0.2 ± 0.1; $P < 0.001$) and that RLX treatment strongly reduces endothelial cell injury (RLX + IR, 1 ± 0.2; IR, 2.9 ± 0.1; $P < 0.001$). Similarly, myocyte injury was significantly increased in the ischemic-reperfused hearts as compared with the sham-operated ones (IR, 2.8 ± 0,1; sham-operated, 0.3 ± 0.2; $P < 0.001$), and RLX treatment strongly reduced myocyte injury (RLX + IR, 0.9 ± 0.2; IR, 2.8 ± 0.1; $P < 0.001$).

Figure 7. Representative electron micrograph of a myocardial cell from the sham-operated rats (group 3) showing normal features. Magnification, ×6300.

Light microscopic examination of cardiac mast cells showed that in the sham-operated hearts these cells were usually rich in secretory granules. In the ischemic-reperfused hearts, mast cells had mostly a few secretory granules and showed clear-cut images of granule exocytosis. At the opposite, in the ischemic-reperfused hearts from the RLX-treated rats, mast cells had numerous secretory granules in their cytoplasms (Figure 10). Morphometric analysis showed that light transmittance across mast cells, which is inversely related to the amount of secretory granules, was significantly higher in the ischemic-reperfused hearts in comparison with the sham-operated ones. Conversely, in the RLX-treated rats, the light transmittance across cardiac mast cells was nearly similar to that of the sham-operated ones (Figure 11).

Discussion

The results of this study show that RLX exerts a significant cardioprotective effect in the ischemic and reperfused rat heart. In fact, systemic administration of RLX results in a marked reduction of the myocardial areas damaged by postischemic reperfusion as well as in a substantial re-

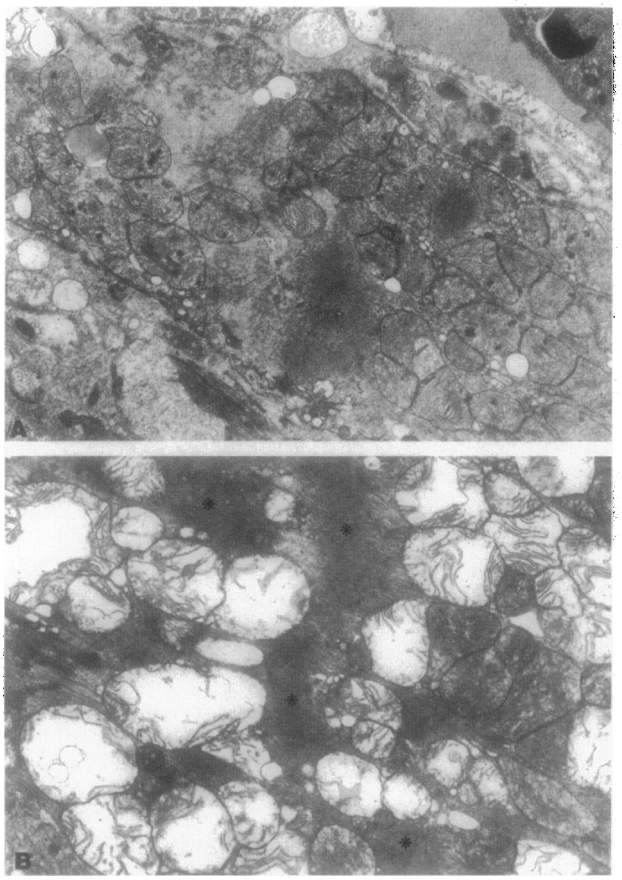

Figure 8. Representative electron micrographs of severely damaged myocardial cells from the ischemic-reperfused hearts without RLX treatment (group 2). **A:** Numerous intramitochondrial dense granules and myofibril contracture. **B:** Marked mitochondrial swelling and disarrangement of myofibrils that appear as dense clusters of myofilaments (**asterisks**). Magnification, ×6300.

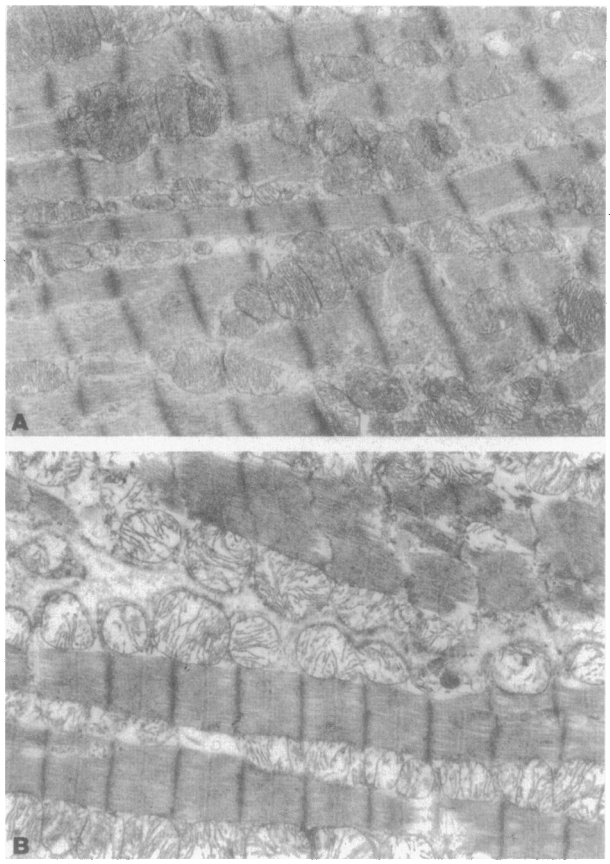

Figure 9. Representative electron micrographs of myocardial cells from the ischemic-reperfused hearts following RLX treatment (group 1). **A:** Normal ultrastructural features. **B:** Moderate mitochondrial swelling without dense granules in the matrix. Magnification, ×6300.

duction of the occurrence of severe ventricular arrhythmias. The current study also shows that RLX acts at multiple levels in the cascade of events that lead to myocardial injury. These events include endothelial dysfunction,[1–5] neutrophil accumulation in the myocardium,[4,6,7,9,11,39–41] and generation of oxygen-derived free radicals by these cells,[4,10–12] platelet and mast cell activation,[8,13,42] and calcium overload that eventually leads to myocardial cell damage and death.[43–46]

In contrast to the ischemic-reperfused hearts of the rats not given RLX, those treated with the hormone showed a marked decrease in the recruitment of neutrophils from the circulation to the myocardium, as indicated by the decrease of MPO activity, a marker for neutrophil accumulation in tissues,[28,29] as well as by morphological analysis.

RLX has been shown to strongly decrease the production of MDA, a lipid peroxidation metabolite,[32] in the ischemic-reperfused rat hearts. This finding indicates that RLX is able to reduce peroxidation of cell membrane lipids induced by oxygen-derived free radicals generated during the reperfusion of the ischemic tissue,[12] thus offering protection to cells that could be damaged by them.

It has repeatedly been shown that calcium leakage occurs during ischemia and reperfusion.[34,35] A loss of calcium homeostasis with excess calcium influx is thought to be a critical event underlying irreversible myocardial injury. RLX has been found to prevent the overload of calcium in the myocardial tissue and to strongly reduce the ultrastructural signs of myocyte alterations like those previously described in conditions of excess intra-

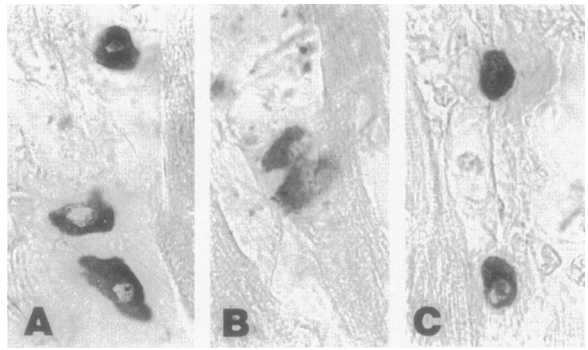

Figure 10. A: Sham-operated heart. Mast cells are intensely stained because of the presence of abundant secretory granules. **B:** Ischemic-reperfused heart. Mast cells are weakly stained because of the paucity of their secretory granules. **C:** Ischemic-reperfused heart following RLX treatment. Mast cells are intensely stained because of a high content of secretory granules. Light microscopy, astra blue staining. Magnification, ×680.

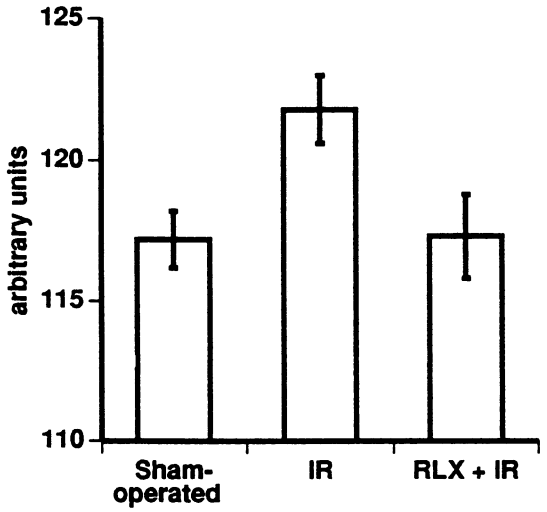

Figure 11. Light transmittance across left ventricular mast cells. Compared with the sham-operated hearts (group 3), the ischemic-reperfused hearts without RLX treatment (IR; group 2) show a significant increase in light transmittance, indicating a decrease in intracellular secretory granules. This effect is abrogated by RLX treatment (RLX + IR; group 1). Values are mean ± SE. Significance of differences (one-way ANOVA, each group, $N = 100$): IR *versus* sham-operated, $P < 0.05$; IR *versus* RLX + IR, $P < 0.05$; sham-operated *versus* RLX + IR, not significant.

cellular calcium, such as hypercontraction of myofibrils, mitochondrial calcification, and cell necrosis.[43–46] Of note, a similar effect of RLX in inhibiting intracellular calcium rise has also been observed in other target cells for this hormone, such as mast cells,[21] vascular smooth muscle cells,[47] and platelets.[20]

An additional contribution to the cardioprotective effect of RLX against ischemia-reperfusion injury may rely on its antiplatelet property. In fact, thrombosis is one of the sequelae of myocardial ischemia-reperfusion-induced damage to the endothelium, and RLX has been shown to reduce the number of circulating platelets[48] and to inhibit platelet activation.[20]

Resident mast cells undergo degranulation during post-ischemic reoxygenation of the heart, thus giving a significant contribution to myocardial ischemia-reperfusion injury through the release of powerful mediators, such as histamine, serotonin, and leukotrienes.[13,14] These substances can affect coronary vascular resistance and permeability and may enhance myocardial damage by increasing tissue edema.[49,50] In turn, edema may hinder restoration of a normal coronary blood flow at reperfusion. Moreover, histamine released by mast cells has been shown to favor neutrophil adhesion to the endothelium[15] and to induce cardiac arrhythmias.[16,17] The results of the current study show that RLX inhibits ischemia-reperfusion-induced mast cell degranulation and hence the release of granule-stored mediators, including histamine, thus possibly contributing to the myocardial salvage. This fits well with the results of our previous studies on serosal mast cells showing that RLX inhibits granule exocytosis and histamine release induced by different stimuli.[21]

In this study, we have shown that RLX can be effective in protecting the heart against ventricular arrhythmias occurring during ischemia and reperfusion. Multiple mechanisms are involved in the development of ventricular arrhythmias. They include generation of oxygen-derived free radicals,[51] peroxidation of myocardial membrane lipids,[52] release of histamine by resident mast cells,[16] and aggregation of platelets.[53] Of note, the current findings and those of previous studies on the effects of RLX on mast cells[21] and platelets[20] indicate that RLX can effectively hamper the above mechanisms, thus providing an explanation for its marked antiarrhythmogenic action observed in the ischemic and reperfused heart.

The current findings show that RLX exerts its cardioprotective effect at very low nanomolar concentrations like those measured in plasma of female rats during pregnancy.[54] This fact allows us to hypothesize that RLX may be a physiological protective agent against heart ischemic disease. This view is supported by the observations that in women, in whom RLX is secreted in the systemic circulation by the corpus luteum during ovarian cycles and even more during pregnancy,[55,56] there is a low incidence of ischemic coronary heart disease during fertile life[57] and a marked increase after menopause[58] coincidentally with cessation of ovarian cycles.

Based on the results of this study, the possibility arises for the future use of RLX or RLX-derived drugs for the prevention and treatment of ischemic heart disease.

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