Antioxidant and Lysosomotropic Properties of Acute D-Propranolol Underlies Its Cardioprotection of Postischemic Hearts from Moderate Iron-Overloaded Rats

JAY H. KRAMER,*,‡,[1] SARAH B. MURTHI,†,[2] ROBERT M. WISE,† I-TONG MAK,*,‡ AND WILLIAM B. WEGLICKI*,‡

*Departments of *Biochemistry and Molecular Biology, Division of Experimental Medicine; †Surgery; and ‡Medicine, The George Washington University Medical Center, Washington, District of Columbia 20037*

The benefits of acute D-propranolol (D-Pro, non–β-adrenergic receptor blocker) pretreatment against enhanced ischemia/reperfusion (I/R) injury of hearts from moderate iron–overloaded rats were examined. Perfused hearts from iron-dextran–treated rats (450 mg/kg/week for 3 weeks, intraperitoneal administration) exhibited normal control function, despite iron treatment that elevated plasma iron and conjugated diene levels by 8.1- and 2.5-fold, respectively. However, these hearts were more susceptible to 25 mins of global I/R stress compared with nonloaded hearts; the coronary flow rate, aortic output, cardiac work, left ventricular systolic pressure, positive differential left ventricular pressure (dP/dt), and left ventricular developed pressure displayed 38%, 60%, 55%, 13%, 41%, and 15% lower recoveries, respectively, and a 6.5-fold increase in left ventricular end-diastolic pressure. Postischemic hearts from iron-loaded rats also exhibited 5.6-, 3.48-, 2.43-, and 3.45-fold increases in total effluent iron content, conjugated diene levels, lactate dehydrogenase (LDH) activity, and lysosomal *N*-acetyl-β-glucosaminidase (NAGA) activity, respectively, compared with similarly stressed nonloaded hearts. A comparison of detection time profiles during reperfusion suggests that most of the oxidative injury (conjugated diene) in hearts from iron-loaded rats occurred at later times of reperfusion (8.5–15 mins), and this corresponded with heightened tissue iron and NAGA release. D-Pro (2 μ*M* infused for 30 mins) pretreatment before ischemia protected all parameters compared with the untreated iron-loaded group; pressure indices improved 1.2- to 1.6-fold, flow parameters improved 1.70- to 2.96-fold, cardiac work improved 2.87-fold, and end-diastolic pressure was reduced 56%. D-Pro lowered total release of tissue iron, conjugated diene content, LDH activity, and NAGA activity 4.59-, 2.55-, 3.04-, and 4.14-fold, respectively, in the effluent of I/R hearts from the iron-loaded group. These findings suggest that the enhanced postischemic dysfunction and tissue injury of hearts from iron-loaded rats was caused by excessive iron-catalyzed free radical stress, and that the membrane antioxidant properties of D-Pro and its stabilization of sequestered lysosomal iron by D-Pro may contribute to the cardioprotective actions of D-Pro. Exp Biol Med 231:473–484, 2006

Key words: iron overload; rat heart; ischemia/reperfusion; D-propranolol; hemodynamic recovery; oxidative tissue injury

Introduction

An association between chronic iron overload and an increased incidence of tissue injury has been reported both clinically (1, 2) and in experimental animal models (3, 4). Clinical hemochromatosis, a disease typified by excessive iron deposits in tissues, has been linked to cirrhosis, multiple endocrinopathies (i.e., diabetes mellitus, hypogonadism, hypoparathyroidism, and hypothyroidism), and cardiac failure. Cardiopulmonary bypass patients receiving blood cardioplegia (5) and anemic patients receiving multiple blood transfusions as part of their normal therapy are often at risk of iron overload (6, 7). In fact, the primary cause of death in transfused β-thalassemia patients is cardiac disease (6, 8–10).

During chronic iron overload, iron accumulates in various tissues, including the liver, kidney, and heart (3, 4). In the heart, iron deposits have perinuclear distributions within muscle fibers, and diffuse fibrosis may also occur, with associated enlargement of heart cavities and wall thinning (11). Intracellular iron is normally stored in ferritin,

Public Health Service grant from the National Institutes of Health RO1-HL66226 supported this study.

[1] To whom correspondence should be addressed at Department of Biochemistry and Molecular Biology, Division of Experimental Medicine, The George Washington University Medical Center, 435 Ross Hall, 2300 Eye Street, N.W., Washington, DC 20037. E-mail: phyjhk@gwumc.edu

[2] Current address: Division of Traumatology & Surgical Critical Care, University of Maryland Medical Center, Baltimore, MD 21201.

Received October 3, 2005.
Accepted December 6, 2005.

1535-3702/06/2314-0473$15.00

a protein whose synthesis is induced by iron influx (1). Iron is subsequently sequestered in acidic (lysosomal or prelysosomal) compartments as iron-loaded ferritin (12). If the storage capacity of ferritin (~2000 iron atoms/mol protein; Ref. 13) is surpassed, the tissue's ability to safely store iron is compromised, allowing release of hemosiderin (partially degraded ferritin) and pathologic amounts of redox-active low molecular weight (LMW) iron; LMW iron can participate in the formation of reactive oxygen species (ROS) capable of inducing oxidative tissue damage (1, 2). LMW iron can catalytically transform (14) superoxide anion ($\bullet O_2^-$) and hydrogen peroxide (H_2O_2) to the more pro-oxidant hydroxyl radical ($\bullet OH$), and lipid hydroperoxide (LOOH) to the more pro-oxidant alkoxyl ($LO\bullet$) and peroxyl ($LOO\bullet$) free radicals. These pro-oxidant species can initiate and possibly propagate membrane lipid peroxidative injury.

The presence of a preexisting iron-overload condition seems to amplify myocardial injury resulting from an imposed oxidative stress, such as ischemia/reperfusion (I/R; Ref. 15). Myocardial ischemia induces metabolic changes that predispose the heart for oxidative injury during reperfusion, and a key event may involve the relocalization of sequestered (lysosomal) ferritin-iron to ectopic binding sites, such as membrane phospholipids. Mobilization of ferritin-iron requires its reduction to the catalytically active ferrous state (Fe^{2+}; Ref. 16); it has been shown that specific metabolic processes ($\bullet O_2^-$ and H_2O_2 generation, reduced flavin mononucleotides, and cellular acidosis) associated with the I/R heart can independently induce iron mobilization (17–20). Once released, this more accessible and enlarged (in the instance of the iron-overloaded tissue) pool of redox-active LMW iron may then participate in the formation of potent oxidants capable of inducing further injury after reintroduction (reperfusion) of molecular oxygen (21).

Because there is no physiologic mechanism for iron excretion in humans (1), metal chelators are commonly used to combat acute iron toxicity (blood cardioplegia during cardiopulmonary bypass; Ref. 5) and chronic overload caused by transfusion (22). The clinical use of the metal chelator, deferoxamine (DFO; also known as trihydroxamic acid), has been shown to significantly decrease morbidity and mortality in compliant transfused patients (1, 7). Experimentally, acute DFO has provided cardioprotection to animal hearts subjected to reperfusion injury (15), confirming a link between pro-oxidant tissue iron and reperfusion injury in this stress model. However, there have been concerns regarding its clinical use (1), including safety issues (7). In view of the pro-oxidant nature of LMW iron, the use of adjunct antioxidant therapy (vitamin C, β-carotene, and vitamin E with or without selenium; Refs. 1, 2, 7, 23, 24) along with DFO chelation has been considered, and showed promise as protective treatments. In addition, certain cardioactive agents also have antioxidant properties independent of their primary pharmacologic action, and may have potential as adjunct therapy during chelation of iron-overloaded individuals. Using isolated biomembranes exposed to exogenous free radicals, we reported (25, 26) that propranolol (Pro) had significant membrane antioxidant activity, which was unrelated to β-adrenergic receptor blockade, because both L-Pro and D-Pro (inactive isomer) forms were equipotent. D-Pro also provided antioxidative protection *in vitro* to oxidatively stressed adult canine ventricular myocytes, cultured bovine aortic endothelial cells, and isolated perfused rat hearts (27–30). It has been shown that lipophilic β-blockers such as Pro, can accumulate intracellularly in various cell types, approaching levels which are 1000-fold higher than their incubation medium concentration (31). This uptake process was shown to be nonstereoselective, and inhibitable by nonspecific lysosomotropic (alkalinization) agents, such as methylamine and NH_4Cl, suggesting the lysosomal compartmentalization of Pro. Agents that induce lysosomal alkalinization (32) would likely enhance the binding interaction between lysosomal iron and ferritin, and attenuate the lysosomal release of redox-active LMW iron during subsequent tissue oxidative stress (I/R).

Although acute D-Pro treatment has been shown to afford cardioprotection to severe I/R-stressed hearts from non–iron-loaded rats (30), its impact on I/R hearts from iron-overloaded animals has not been reported. In iron-overloaded rodent models, defects in basal cardiac function (depressed heart rate; positive differential left ventricular pressure (+dP/dt); left ventricular systolic pressure (LVSP); left ventricular developed pressure (LVDP); and increased diastolic pressure and/or abnormal electrocardiographs) have typically been observed (33–36) using relatively high iron-loading doses (800-2500 mg Fe/kg/week) and/or after prolonged iron exposure times (4–20 weeks). In the current study, the severity of the *in vivo* iron-loading protocol (450 mg Fe-dextran/kg/week, administered intraperitoneally [ip] for 3 weeks) was adjusted to avoid issues concerning the use of functionally impaired rat hearts in subsequent I/R stress experiments. Postischemic hearts from rats exposed to this moderate iron-overloading regimen were shown to exhibit significantly greater hemodynamic dysfunction and oxidative tissue injury compared with equally stressed untreated hearts. Comparisons of detection time courses for several indices of injury implicate the potential involvement of lysosomal iron in the cascade of injurious events during reperfusion. In addition, we present evidence that acute D-Pro pretreatment effectively reduced the additional reperfusion injury experienced by these hearts, and that this protection is likely the consequence of the agent's membrane antioxidant and lysosomotropic (influence on sequestered iron) properties, rather than its β-receptor–blocking actions.

Materials and Methods

Chemicals. All dry chemicals and solvents were obtained either from Fisher Scientific (Pittsburgh, PA) or

from Sigma Chemicals (St. Louis, MO). Lactate dehydrogenase (LDH) assay kits were from Sigma Chemicals. All buffers and assay solutions were prepared with deionized, double distilled water, as previously described (37), and levels of trace metals (atomic absorption spectroscopy; Shimadzu AA-6200; Columbia, MO) were below the limits of detection.

Animal Assurance. All animal experiments were guided by the principles for the care and use of laboratory animals, as recommended by the US Department of Health and Human Services and approved by The George Washington University Animal Care and Use Committee.

Iron Treatment *In Vivo*. Male Sprague-Dawley rats (300–400 g) were maintained on a commercially available (Harlan Teklad, Madison, WI) nutritionally balanced diet (containing 42 ppm iron) and were given free access to deionized, distilled water. We used previously described iron-dextran loading procedures (15, 16) that have not been associated with gross changes in animal body weight, eating habits, or behavior. Anesthetized (2–5% isoflurane, inhalation anesthesia chamber; EZ Anesthesia, Palmer, PA) rats received two ip injections of iron dextran solution (100 mg/ml; Sigma Chemicals) per week, resulting in 450 mg iron/kg/week for up to 3 weeks. Iron loading (in tissue and plasma) was verified by atomic absorption spectroscopy. Controls received equal injection volumes of 10% sodium dextran. Animals were given free access to deionized water and received normal diet. During the fourth week, anesthetized rats were sacrificed for the postischemia studies, and blood samples were collected for subsequent use in iron and conjugated diene determinations.

Global I/R Model and Drug Treatment. The isolated perfused working rat heart and global I/R models were described previously (37, 38). Hearts (nonpaced) from anesthetized rats were cannulated by the aorta (afterload = 80 mm Hg) to a working heart perfusion system within 60 secs of excision, and were subsequently converted to working mode perfusion by cannulating the left pulmonary vein. Hearts were then exposed to 45 mins of non-recirculating stabilization (preischemic control) with physiologic Krebs-Henseleit buffer (KHB; gassed with 95% O_2:5% CO_2, pH 7.4; 37°C) containing 1.25 m*M* $CaCl_2$ and 10.0 m*M* glucose; baseline functional/hemodynamic parameters and biochemical (effluent LDH, *N*-acetyl-β-glucosaminidase [NAGA], iron, and conjugated dienes) measurements were taken at the end of this period. In parallel studies, 2 μ*M* D-Pro (in 0.9% saline) was infused (0.5 ml/min for 30 mins, yielding 30 nmol final) *via* a side arm connected to the aortic perfusion line after an initial 15-min stabilization period. The influence of *in vivo* treatment with iron-dextran on the susceptibility of hearts to I/R stress *in vitro*, was assessed in hearts subjected to 25 mins of global no-flow ischemia followed by 30 mins of reperfusion (0- to 15-min interval in Langendorff mode and 15- to 30-min interval in working mode) under preischemic conditions; the measurements were repeated for estimates of postischemic injury.

Heart Function/Hemodynamics. Coronary flow rate, aortic output, and various pressure indices (Statham Gb transducer for LVSP, left ventricular end-diastolic pressure [LVEDP], LVDP, and +dP/dt) were monitored during perfusion (37). Percent recovery of each parameter and cardiac pressure-volume work was determined from the ratio of the value measured at the end of reperfusion to that obtained during the preischemic control period.

Iron Determinations. During the last 5 mins of control perfusion and the initial 15 mins of Langendorff reperfusion, coronary effluent samples were collected sequentially for total iron determination (2 ml straight) by flame emission atomic absorption spectroscopy. Reperfusion time courses were constructed after normalizing values to their preischemic control levels, and area integration of time courses was used to determine total effluent iron content during reperfusion. Plasma iron content was assessed by atomic absorption using acidified diluted samples (1:10 in trace-metal grade 0.5 *N* HNO_3). Each sample value was an average of four separate readings per sample.

Conjugated Diene Determinations. During the last 5 mins of control perfusion and the initial 15 mins of Langendorff reperfusion, coronary effluent samples (1 ml) were collected sequentially for conjugated diene determinations. After chloroform:methanol (C:M) extraction, drying under N_2 gas, and reconstitution into 1 ml cyclohexane, extracts were read by UV spectroscopy (Beckman DU-8; Somerset, NJ) at 233 nm. Reperfusion time courses were constructed after normalizing values to their preischemic control levels, and area integration of time courses was used to determine total effluent conjugated diene levels during reperfusion. Plasma conjugated diene levels were assessed after C:M extraction/cyclohexane reconstitution of 0.5 ml plasma.

NAGA and LDH Determinations. During the last 5 mins of control perfusion and the initial 15 mins of Langendorff reperfusion, coronary effluent samples (50 μl) were collected sequentially for NAGA activity (lysosomal acid hydrolase) determination by a spectrocolorimetric (410 nm) assay (39) and for LDH activity (a tissue injury marker) using spectrophotometric (340 nm) assay kits (Sigma Chemicals; Ref. 37). Reperfusion time courses were constructed after normalizing values to their preischemic control levels, and area integration of time courses was used to determine total effluent NAGA and LDH activities during reperfusion.

Effect of Pro on Lysosomal Stability *In Vitro*. The direct dose-dependent effects of D,L-Pro on lysosomal stability during exogenous free radical exposure ($\bullet O_2^-$ and •OH generating system) was assessed *in vitro* using enriched (~20-fold) lysosomal preparations obtained by differential centrifugation (20,000 *g* pellet; Ref. 39). Typical latency of the enriched preparations was 85%.

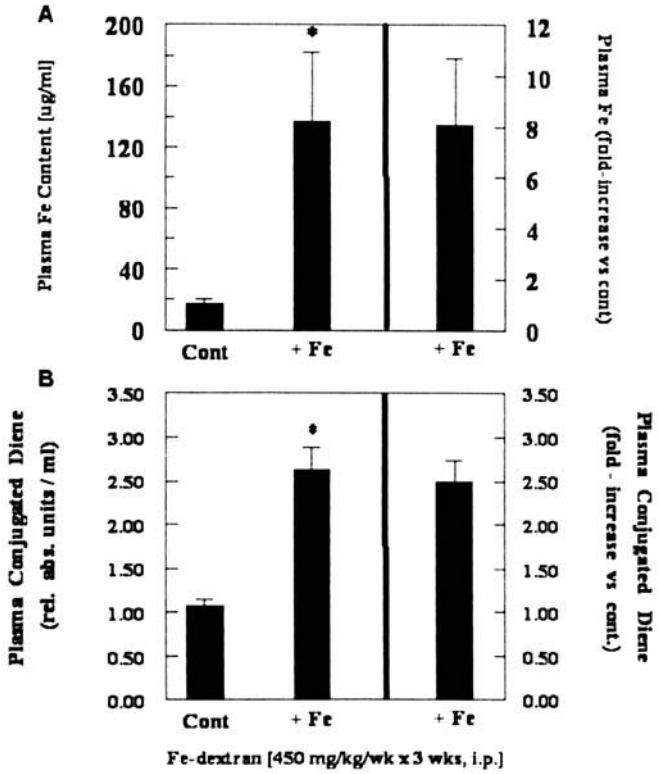

Figure 1. Effects of chronic iron overload on rat plasma iron (A) and conjugated diene (B) content. Animals received biweekly ip injections totaling 450 mg iron-dextran/kg/week (or sodium dextran as control) during 3 weeks, and blood samples were collected at sacrifice for use in iron (atomic absorption) and conjugated diene (UV spectroscopy) determinations. Values are means ± SE of five or six rats. $^*P < 0.05$ versus control.

Hepatic tissue from non–iron-loaded rats was the source of lysosomes because of difficulties in obtaining reasonable yields of enriched material from cardiac tissue, and to avoid concerns related to the fragility of lysosomes prepared from iron-loaded rat tissue. After 10 mins of preincubation (37°C) of lysosomes (200 μg protein/ml) with or without Pro (20, 50, and 100 μ*M*), an exogenous free radical–generating system (25 μ*M* $FeCl_3$ chelated with 250 μ*M* ADP + 83 μ*M* dihydroxyfumaric acid; 37°C; Ref. 40) was introduced; NAGA activity in the incubation medium was assessed after the 30-min exposure period in the presence or absence of Triton X-100 (total NAGA activity) to obtain values for percent free NAGA activity (means ± SE of three preparations).

Statistical Approaches. Analysis of variance (ANOVA) was used for comparison of several means and the Tukey test was used for all paired mean comparisons. Significance was considered at $P < 0.05$.

Results

The moderate iron-loading regimen (450 mg Fe-dextran/kg/week for 3 weeks, ip) resulted in an 8.1-fold increase in plasma iron content and a 2.5-fold elevation in conjugated diene levels (Fig. 1A and B), indicating that these animals were experiencing oxidative stress *in vivo*. However, this did not translate into significant changes in any of the basal (preischemic) hemodynamic parameters monitored in perfused working hearts from the iron-treated group compared with the untreated group (Table 1). By contrast, hearts from the iron-loaded group proved to be much more susceptible than from the untreated group to functional/hemodynamic injury if I/R stress was imposed. Hearts from the iron-treated group displayed substantially lower recoveries of coronary flow rate (38% lower); aortic output (61% lower); cardiac work (55% lower); LVSP (13% lower); LVDP (15% lower; not shown); and dP/dt (41% lower); and showed increased LVEDP (6.5-fold), compared with the equally stressed untreated group (Fig. 2).

Acute pretreatment of hearts from iron-loaded rats with 2 μ*M* D-Pro dramatically improved postischemic recovery of each of these parameters: coronary flow rate was 1.7-fold higher; aortic output was 2.96-fold higher; cardiac work was 2.87-fold higher; LVSP was 18.6% higher; LVDP was 55% higher (data not shown); dP/dt was 60% higher; and LVEDP was 59% lower than I/R hearts from iron-loaded rats (Fig. 2). D-Pro pretreatment of I/R hearts from non–iron-loaded animals only provided significant ($P < 0.05$) protection with respect to LVEDP (36% lower) compared with control. Although recovery of pressure indices were improved with acute D-Pro pretreatment of the iron-loaded group, the greatest protective effects occurred with respect to the myocardial flow parameters (coronary flow rate and

Table 1. Fe-Dextran Treatment *In Vivo* Did Not Alter Hemodynamic Parameters of Perfused Preischemic Rat Hearts[a]

Preischemic perfusion indices	Na-dextran	Fe-dextran
Cardiac work (kg-m/g dry wt/min)	0.426 ± 0.004	0.404 ± 0.01
Cardiac output (ml/min)	59.7 ± 3.7	56.7 ± 2.2
Coronary flow rate (ml/min)	17.7 ± 1.8	18.1 ± 1.5
LVSP (mm Hg)	113.6 ± 3.8	113.4 ± 1.6
LVEDP (mm Hg)	4.23 ± 0.28	4.05 ± 0.35
LVDP (mm Hg)	109.0 ± 3.6	110.0 ± 3.0
Left ventricular dP/dt (mm Hg/sec)	5982 ± 126	5813 ± 1.8

[a] Rats received 450 mg Fe-dextran/kg/week (or Na-dextran) by ip injection for up to 3 weeks before isolated working heart perfusion. All values are means ± SE of five or six hearts; no significant differences were detected between groups.

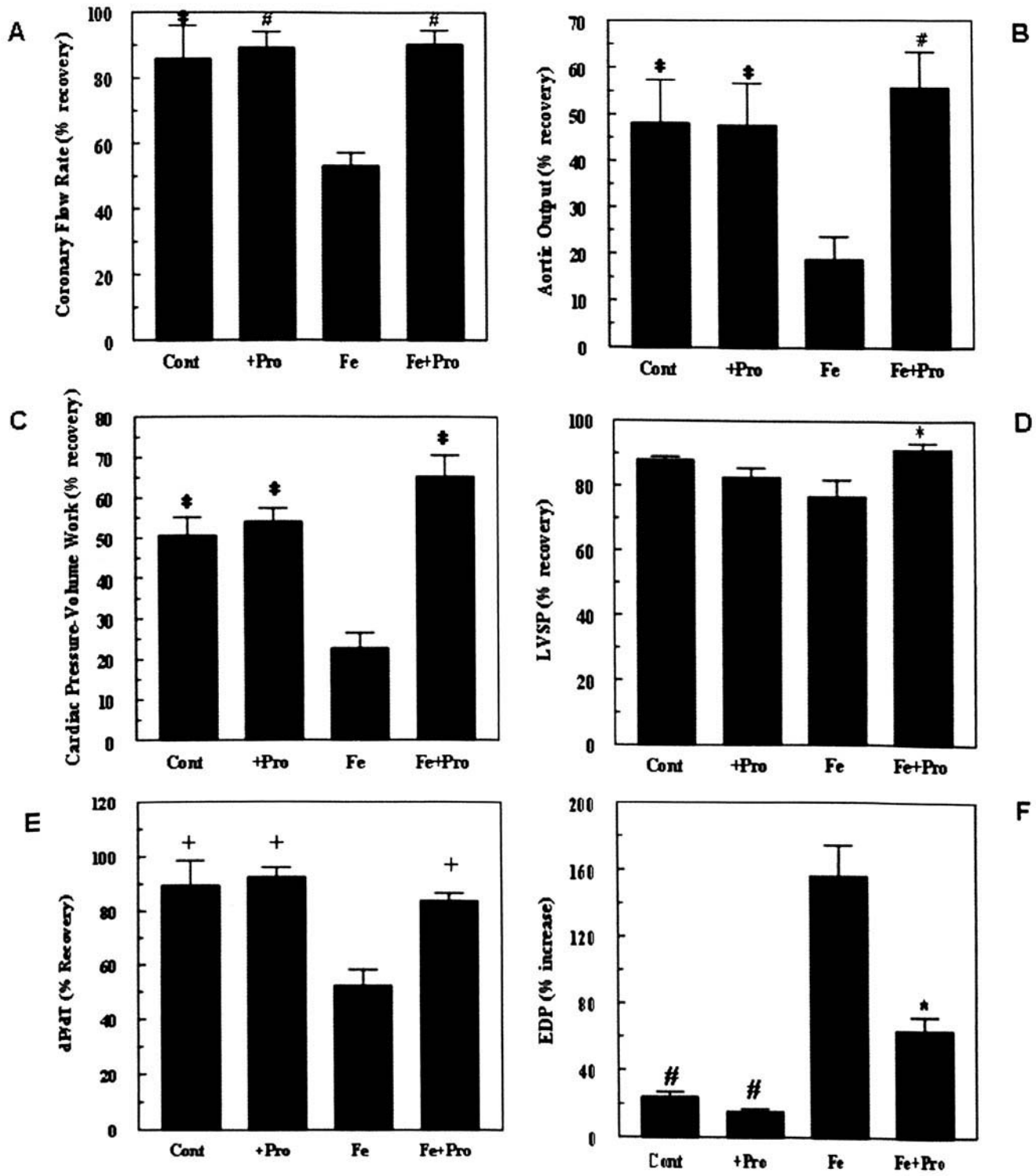

Figure 2. Effects of acute D-Pro pretreatment on postischemic recoveries of (A) coronary flow rate; (B) aortic output; (C) cardiac pressure-volume work; (D) LVSP; (E) dP/dt; and (F) end-diastolic pressure (EDP) of hearts from Fe-dextran– or Na-Dextran (control)–loaded rats. After 3 weeks of the loading regimen, isolated perfused working rat hearts were exposed to 30 mins of 2 μM D-Pro pretreatment before 25 mins of normothermic global ischemia and 30 mins of reperfusion. Values for each parameter at the end of reperfusion were compared with those recorded during the preischemic period to obtain recovery values. Values are means ± SE of five or six hearts. $^*P < 0.05$; $+P < 0.02$; $\#P < 0.01$, versus iron alone.

Table 2. Fe-Dextran Treatment *In Vivo* Did Not Alter Oxidative and Tissue Injury Indices of Preischemic Rat Hearts[a]

Preischemic indices (effluent)	Na-Dextran	Fe-Dextran
Conjugated dienes (Rel. Abs. Unit/ml/g)	0.129 ± 0.010	0.122 ± 0.028
Released iron (μg/ml/g)	0.084 ± 0.030	0.099 ± 0.028
LDH activity (U/l/g)	1.106 ± 0.320	2.010 ± 0.800
NAGA activity (Rel. Abs. Unit/ml/g)	0.098 ± 0.012	0.082 ± 0.022

[a] Rats received 450 mg Fe-dextran/kg/week (or Na-dextran) by ip injection for up to 3 weeks before isolated working heart perfusion. Effluent samples taken during preischemic perfusion were used to assess the parameters. All values are means ± SE of five or six hearts; no significant differences were detected between groups. Rel. Abs. Units, relative absolute units.

aortic output), and this was also reflected in the substantial improvement of postischemic cardiac pressure-volume work (functional recovery index).

Normally perfused hearts from iron-loaded rats did not exhibit significant changes in preischemic markers of oxidative stress and tissue injury compared with untreated rats (Table 2), but the enhanced hemodynamic/functional depression displayed by I/R-stressed hearts from the treated group was associated with substantially higher levels of these end points. Figure 3 compares total postischemic production of key markers of oxidative stress and tissue injury detected in the effluent of the different I/R-stressed heart groups. The iron-loaded group exhibited significantly higher total postischemic LDH activity (2.4-fold); conjugated diene levels (3.5-fold); iron release (5.6-fold); and lysosomal NAGA activity (3.45-fold) compared with the unloaded group. Pretreatment of I/R hearts from iron-loaded rats with 2 μ*M* D-Pro afforded significant protection, as shown by the dramatic decreases in the levels of each parameter (67%, 61%, 78%, and 76% lower for LDH, conjugated dienes, iron, and NAGA, respectively). D-Pro did not provide significant additional protection to I/R hearts from non–iron-loaded rats. The reperfusion time-course profiles (Fig. 4A–D) reveal the maximal detection times for these indices, and implicate a potential sequence of damaging events in this model. Loss of plasmalemmal (sarcolemmal) integrity seems to be an early event in the I/R-stressed iron-loaded heart model, as suggested by the relatively massive early release (peak at 3–5 mins) of LDH (cytosolic enzyme) from the reperfused heart tissue. By contrast, most, but not all, of the postischemic conjugated diene formation and iron release occurred during later times (7–15 mins) of reperfusion. Interestingly, the postischemic NAGA release profile bore a striking resemblance to that observed for conjugated diene and iron, with most of the release occurring during the later reperfusion period (7–15 mins). This suggests that redox-active iron derived from destabilized lysosomes may have contributed to oxidative tissue injury during the later stage(s) of reperfusion. Acute D-Pro substantially lowered the iron treatment–induced increases in markers of oxidative and tissue injury during the early (0–6 mins) and later (7–15 mins) phases of reperfusion (Fig. 4A–D).

The direct effect of Pro on lysosomal stability during 30 mins exposure to exogenous free radical stress was assessed *in vitro* using rat hepatic tissue lysosomal preparations. Exposure to the free radical–generating system caused a substantial loss of lysosomal latency (61 ± 3.0% free NAGA) compared with control preparations (18.1 ± 0.89%). D,L-Pro dose-dependently (20, 50, and 100 μ*M*) preserved lysosomal integrity during oxidative stress, as shown by significant ($P < 0.02$) declines in free NAGA released from drug-pretreated preparations (46 ± 1.6%, 39.3 ± 1.4%, and 31 ± 1.6% free NAGA, respectively) compared with the free radical–stressed untreated preparation.

Discussion

Unlike our iron-overload study (41), which caused basal rat heart dysfunction, the moderate regimen used here did not alter hemodynamic parameters (Table 1), or cause overt changes in markers of oxidative stress and tissue injury (Table 2) during normal heart perfusion. The moderate regimen did induce oxidative stress *in vivo*, as suggested by the 8.1-fold rise in plasma iron and the 2.5-fold increase in plasma conjugated diene content (Fig. 1). Thus, this moderate procedure did not achieve the severity threshold of oxidative stress *in vivo*, which culminated in irreversible basal dysfunction during normal heart perfusion.

Hearts from moderately iron-overloaded rats were predisposed to I/R stress. This was evidenced by the poor recoveries of postischemic myocardial flow parameters, cardiac work, dP/dt, and end-diastolic pressure (Fig. 2). The further decrease in dP/dt suggests that the velocity of left ventricular contraction was severely compromised by iron overload, and the large increase in end-diastolic pressure is indicative of ventricular stiffening (poor relaxation). Our findings are similar to those of van der Kraaij *et al.* (15), who reported enhanced reperfusion dysfunction and tissue injury to iron-overloaded rat hearts in the absence of basal cardiac dysfunction. By contrast, Pucheu *et al.* (42) failed to observe additional postischemic functional injury in their iron-overloaded rat model. Differences in iron-loading protocols (~200 mg Fe-dextran/kg/week for 5 weeks vs. 450 mg/kg/week for 3 weeks in the present study), heart perfusion methodologies (constant flow vs. working heart in

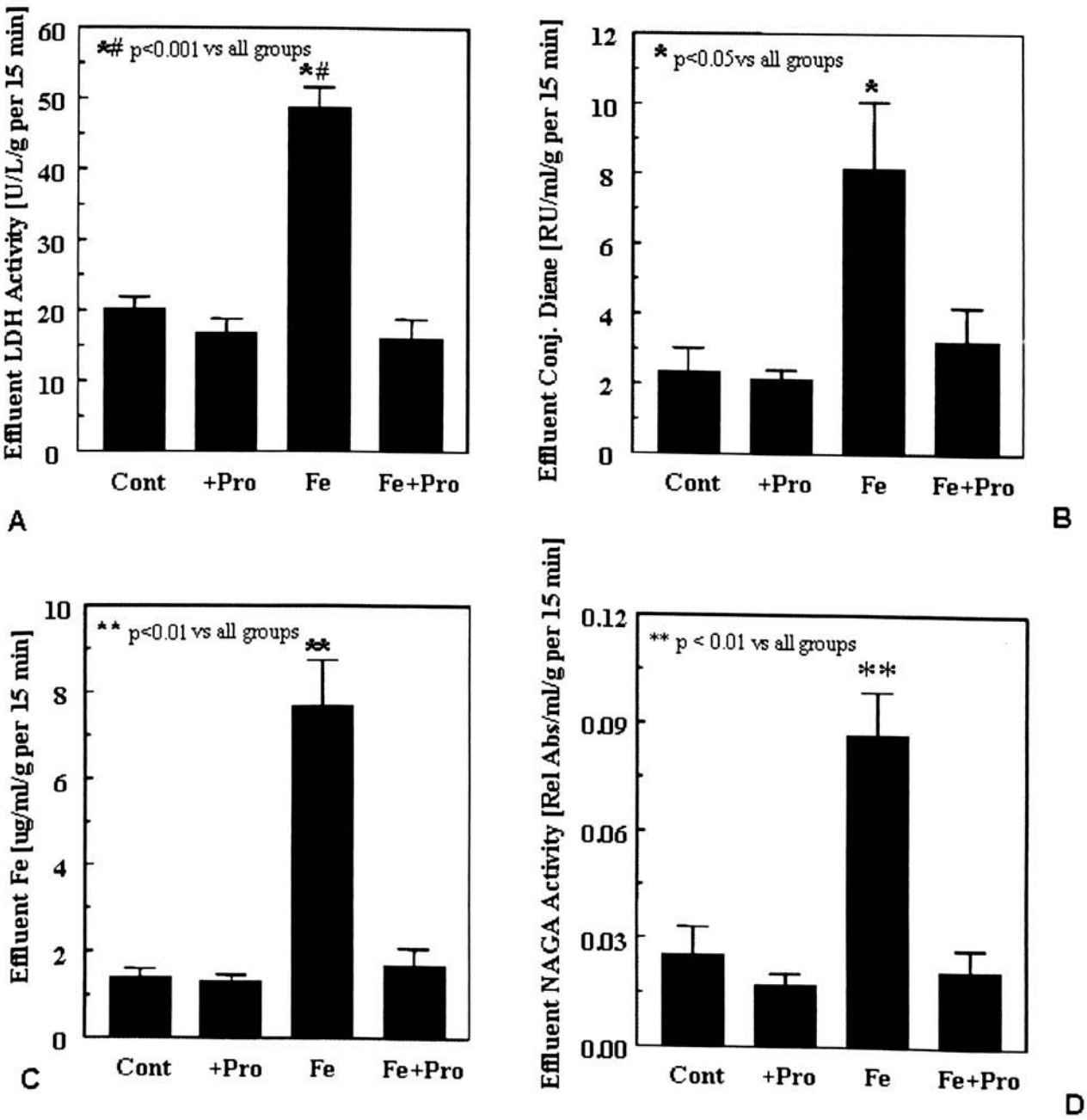

Figure 3. Effects of acute D-Pro pretreatment on total postischemic production of effluent: (A) LDH activity; (B) conjugated diene levels; (C) iron content; and (D) lysosomal NAGA activity of hearts from Fe-dextran– or Na-dextran (control)–loaded rats. After 3 weeks of the loading regimen, isolated perfused working rat hearts were exposed to 30 mins of 2 μ*M* D-Pro pretreatment before 25 mins of normothermic global ischemia and 30 mins of reperfusion. Effluent samples were collected sequentially during reperfusion for the respective analytical assays, and were normalized to preischemic levels. Total levels were determined by area integration of time courses. Values are means ± SE of five or six hearts. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{*\#}P < 0.001$, versus all groups.

our study), and durations of global ischemia (15 mins vs. 25 mins in our study), may partly explain the dissimilar results. Indeed, the importance of ischemic duration was shown in another report by this group (43), which revealed a direct association between duration and the progressive rise in cytosolic iron content (30 mins > 20 mins > 10 mins of ischemia) in non–iron-loaded (control) rat hearts. In this instance, increasing the ischemic duration may cause progressively greater postischemic iron release, higher iron-mediated free radical production, and enhanced dysfunction. In this light, we previously demonstrated: (i) a direct relationship between *in vivo* iron loading doses and elevations in iron-catalyzed production of lipid peroxidation (LPO)-derived LO• radicals from 30-min I/R rat hearts (44); and (ii) direct correlations of rising LO• production with lower functional recovery ($r = -0.83$ to -0.87; Ref. 30), and with increasing cardiac iron release ($r = 0.87$; Ref. 44) after longer (15–60 mins) ischemic episodes in control hearts. Thus, differences in experimental conditions can influence the impact of endogenous iron on cardiac tolerance to I/R

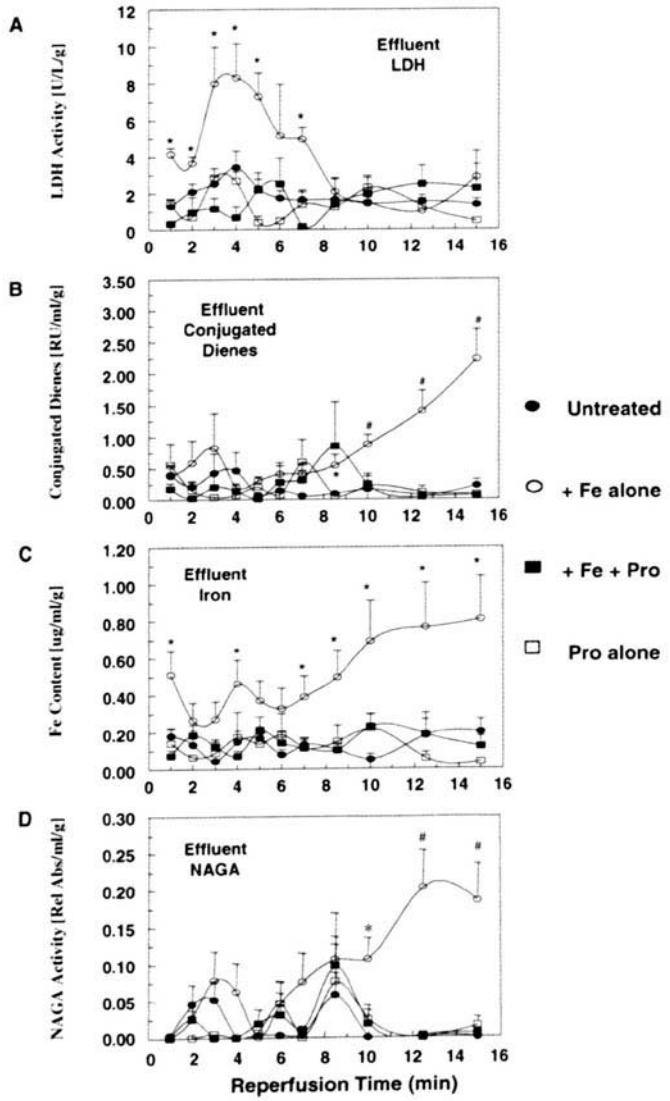

Figure 4. Effects of acute D-Pro pretreatment on postischemic detection times of (A) LDH activity; (B) conjugated dienes; (C) iron; and (D) lysosomal NAGA activity in effluent from hearts of Fe-dextran– or Na-dextran (control)–loaded rats. The same conditions as in the legend for Figure 3 were used. Values are means ± SE of five or six hearts. $^*P < 0.05$; $\#P < 0.02$, versus untreated.

stress, possibly by altering the availability of redox-active iron during reperfusion.

Intracellular redistribution and mobilization of LMW iron after prolonged global ischemia ($\geq$ 20 mins in rat) may partially dictate the severity of reperfusion injury (43, 45). Some investigators proposed that endogenously produced $\bullet O_2^-$ (18) or H_2O_2 (19) may mobilize iron from intracellular proteins such as ferritin or myoglobin, respectively. However, this may not explain the rise in cardiac cytosolic iron content during ischemia (43), because ROS generation would largely take place during early reperfusion (38, 44). Alternatively, the redistribution of stored iron during ischemia may involve tissue accumulation of reducing equivalents and acidification (43, 44), which occur because of a shift from aerobic to anaerobic metabolism. Funk *et al.* (17) showed that reduced flavin mononucleotides provoked iron release from ferritin at neutral pH, and acidification further amplified release. We reported (44) that control rat hearts exposed to acidotic (pH 6.4) preconditioning exhibited substantial iron release (13-fold > nonpreconditioned) into coronary effluent, which was comparable to that seen (11.4-fold higher) with ischemic preconditioning. The total amount of iron (1.21 ± 0.13 μg/ml/g [± SE]; $n = 3$) released by acidotic preconditioning (per 20-min perfusion) was remarkably similar to that of 25-min I/R control hearts (Fig. 3), implicating an involvement of acidosis in I/R-induced iron release. Preconditioning before severe I/R decreased LO• production nearly 75% versus nonpreconditioned (44); thus, decreasing the availability of LMW iron before prolonged ischemia may reduce reperfusion injury.

Acidosis may impact the severely ischemic heart in ways unrelated to iron status (protons compete with calcium to impair contractility, inhibit calcium channels, decrease glycolytic flux by inhibiting phospho-fructokinase; Ref. 46; or inhibit citrate synthase activity; Ref. 47). However, some literature suggests that acidosis without ischemia may not be damaging (48), or cause irreversible loss of mechanical function or metabolic viability (47). Conversely, acidosis mobilizes cardiac iron (44), and this may underlie much of the enhanced irreversible oxidative injury and mechanical dysfunction observed in iron-loaded I/R rat hearts.

Because lysosomes are principal cellular storage sites for iron and contain several hydrolytic enzymes (22), their stability during I/R and their contribution toward the development of irreversible injury has been widely studied. The reported duration of ischemia necessary for detection of instability (based on lysosomal enzyme release) has varied from 30 mins (49) to more than 3 hrs (50). However, because a rise in cytosolic iron content may occur as early as after 20 mins of ischemia (43), an initial iron mobilization from partially destabilized lysosomes may precede more severe lysosomal disruption that permits leakage of hydrolytic enzymes after 30 mins (49). Preexisting iron overload *in vivo* may amplify or accelerate this series of events in I/R hearts (51). In our study (Fig. 3), I/R-stressed iron-loaded hearts exhibited substantially greater release of lysosomal NAGA activity into effluent versus control hearts, and this was associated with elevations in total effluent LDH activity, conjugated diene levels, and iron content. The detection time course data (Fig. 4) suggest that plasmalemmal (sarcolemmal) integrity was lost early during reperfusion, as evidenced by the release of LDH (3–5 mins), which is consistent with observations for control I/R rat hearts (52). Although the time course of LDH had monophasic characteristics, conjugated diene, iron, and NAGA released into the effluent all displayed multiphasic profiles. In agreement with others (43, 45), significant ($P < 0.02$) losses of tissue iron (2.2-fold > preischemic levels) from control hearts occurred within the first minute of reperfusion, and was even more dramatic (5.2-fold higher) in the iron-loaded group. This initial loss may be a consequence of

tissue washout, but its release at the onset of reperfusion may coincide with an early production (by 20 secs) of $\bullet O_2^-$, which we (53) have shown occurred in I/R-stressed control rat hearts. In contrast with control hearts (43, 45), iron-loaded hearts exhibited a more distinct peak of iron release at 3–5 mins of reperfusion, and displayed a progressive rise in effluent iron between 7 and 15 mins. Our detection (38) of •OH spin adducts by electron spin resonance (ESR) spectroscopy at 3 mins (peak) of reperfusion in I/R-stressed control hearts might explain the subsequent (≥3 mins) oxidative injury, plasmalemmal hyperpermeability, and lysosomal instability observed in the iron-loaded I/R heart. Use of free-radical detection methodology and/or ROS-specific antioxidants in the iron-loaded I/R rat heart model would be needed to confirm these contentions.

Our findings suggest that at least two pools of redox-active iron were available to participate in the oxidative toxicity mechanism during I/R. The smaller iron pool may have a cytosolic distribution (at or near macromolecular or biomembrane sites) during ischemia (43), because it is released early (within 1 min) during reperfusion, and preceded the earliest peak detection (after 3 mins) of lysosomal disruption and plasmalemmal injury. This cytosolic pool may have originated from the ferritin-iron complex of mildly destabilized lysosomes (before substantial hydrolytic enzyme leakage) during the ischemic episode, but the metabolic events (tissue acidosis, free radicals, and reducing equivalents; Ref. 44) that elicited iron mobilization remain unresolved. ESR and spin trapping enabled our detection of endogenous free radicals in the *in vivo* regionally ischemic canine heart (LO• and alkyl [L•] radicals; Ref. 54) and buffer-perfused global ischemic rat heart (•OH and L• radicals; Ref. 55) models in the absence of reperfusion. Our observations (55) of free-radical production during the first 25 mins of global rat heart ischemia, and its absence thereafter, led us to conclude that tissue oxygen levels were sufficient to support this production only during short-to-moderate durations of ischemia. This study does not refute the notion that most endogenous free-radical production occurs during reperfusion, but presents the possibility that low-level production may take place during early stages of ischemia. In this light, we propose a mechanism of lysosomal iron mobilization during ischemia that involves a combined tissue acidosis–ROS interaction. In support of this proposal are reports that: (i) cardiac acidosis does occur rapidly (onset within 5 secs) and is progressive during ischemia (tissue pH fell 0.66, 1.04, and 1.41 U at 15, 30, and 60 mins; Ref. 56); (ii) anoxia in the absence of cardiac acidosis induces accumulation of reducing equivalents, but not a rise in cytosolic iron content (16); and (iii) *in vitro*, acidified (pH 6.0) incubation medium enhanced the rate and magnitude of rat liver lysosomal membrane LPO (substantial within 10 mins) during exogenous oxy-radical exposure, and this preceded notable increases (>15 mins) in free NAGA release (40). The acidosis-ROS mechanism does not minimize the contribution of ischemia-induced accumulation of reducing equivalents, but highlights the possibility that lysosomal iron mobilization may occur in its absence (16).

The larger second wave of iron release (3–5 mins and 8.5–15 mins) is much more exaggerated in iron-loaded I/R hearts compared with control hearts (43, 45), and may originate from iron-loaded lysosomes that become severely destabilized because of oxidative stress at later times of reperfusion. The transient nature of •OH spin adduct detection (0.5–7 mins) in I/R control rat hearts (38) led to our conjecture that the progressive elevations observed thereafter (>7 mins) for conjugated-diene content, iron, and NAGA release may be caused by propagation of the cardiac membrane LPO chain reaction (30). Indeed, the biphasic time courses for LOOH formation in control I/R rat hearts (57) and for iron-catalyzed LO• production (44) from iron-treated I/R rat hearts (maximums: 3–6 mins and 8.5–12.5 mins reperfusion for 3 *in vivo* iron doses) were very similar to the detection profiles of the second wave of liberated iron, NAGA, and conjugated dienes. The biphasic LO• detection pattern may reflect a time lag in the occurrence of oxidative injury to different anatomic regions of the I/R heart; however, similar biphasic patterns of LO• production were reported for anoxia/reoxygenated cardiocytes and endothelial cells (30), thus, supporting an interplay between the second wave of tissue (lysosomal) iron release and development of oxidative injury during later times of reperfusion.

Basic amine-containing compounds, such as Pro, raise lysosomal pH and should diminish the availability of lysosomal iron during I/R-stress of iron-loaded hearts. The ethanolamine side chain of Pro, which contains an amino moiety, confers the lysosomotropic properties of Pro. In cell models, the lysosomotropic effect of Pro was seen at low concentrations (10^{-7} to 10^{-9} *M*) compared with methylamine or NH_4Cl (10^{-2} *M*), and depended on the cell's uptake mechanism and ability to accumulate Pro within lysosomes (31). We showed lysosomal accumulation of fluorescently tagged Pro and an associated pH elevation (alkalinization) in cultured aortic endothelial cells (58). Cramb (31) showed that cells lacking lysosomes (red blood cells and platelets) do not take up significant levels of Pro, whereas endothelial cells accumulate Pro more rapidly than adult cardiocytes. These finding are significant when considering: (i) histologic evidence that iron-dextran loading in rats showed myocardial iron enrichment primarily within endothelial cell vesicles and vascular pericytes (16); and (ii) our x-ray microanalyses showing that the amount of iron lost from vascular endothelium during acidotic or ischemic preconditioning of control rat hearts was far greater than that seen for tissue myocytes (44). These vascular-enriched iron sites may amplify I/R injury, and may explain why D-Pro, which preferentially target these sites, affords significant protection.

The cardioprotective efficacy of D-Pro is partly dependent on pretreatment duration before oxidative stress. Adult

cardiocytes pretreated for 10 mins with 2 μ*M* Pro were not protected against anoxia/reoxygenation injury (29), yet were protected after a 2-hr preincubation. By contrast, maximal antioxidant protection to oxidatively stressed endothelial cells was provided by 30 mins of D-Pro preincubation (58). This differential response suggested that the protective effect of Pro required active cellular uptake (endocytosis) and that this process was superior in endothelial cells versus cardiocytes. We found (30) that control rat hearts pretreated for 30 mins with 2 μ*M* D-Pro were substantially protected against reperfusion injury after 40 mins of ischemia. Given the similar pre-exposure times needed for cardiac and endothelial cell protection, we concluded that Pro's efficacy related to its preferential accumulation within lysosomes of the vascular endothelium (58).

In the current study, 30-min pretreatment with 2 μ*M* D-Pro significantly reduced reperfusion injury of 25-min ischemic iron-loaded rat hearts. It improved recovery of pressure indices, flow parameters, and cardiac work (Fig. 2), and caused dramatic reductions in effluent levels of reperfusion injury markers (Figs. 3 and 4). D-Pro afforded only minor protection to control I/R hearts, except for a significant ($P < 0.05$) decline in end-diastolic pressure. These data may reflect our use of less-severe global ischemia (25 mins) in the current study compared with our previous work (40 mins; Ref. 30), and the expected differences in the level of redox-active iron participation (40 mins > 25 mins ischemia) in the I/R injury process of both heart groups (44).

The widely recognized membrane antioxidant property of D-Pro undoubtedly contributed to its cardioprotective effects. Pro exhibits relatively potent antioxidant properties unrelated to β-receptor blockade (L-Pro and D-Pro forms are equipotent) as long as its naphthoxyl linkage is maintained (25). D-Pro is ineffective as a primary free radical (•OH, $•O_2^-$) scavenger (26), and its antioxidant action may resemble the membrane LPO-chain–breaking mechanism of vitamin E (30). D-Pro (EC_{50} = 6.7 μ*M*) interrupted L• radical formation during LPO in an ESR–2-methyl-2-nitrosopropane spin-trapping study using exogenous free radical–stressed ventricular sarcolemma (26). Preincubation with D-Pro was required to achieve this effect, indicating a drug-biomembrane interaction was essential. Because D-Pro interrupts LPO at a stage preceding LO• formation, this may explain its inhibitory effect on I/R rat heart LO• generation (30).

In the current study, the antioxidant effect of Pro was observed with respect to *in vitro* lysosomal stability. Lysosomes exposed to exogenous free radicals displayed a substantial loss of latency (3.3-fold rise in free NAGA) versus controls. Pretreatment with 20–100 μ*M* Pro improved lysosomal integrity (25%–49% lower free NAGA) during oxidative stress versus the untreated free radical–stressed group. This data reinforced our view that Pro's protection of iron-loaded I/R hearts may relate to both its lysosomal membrane-stabilizing influence (antioxidant) and to its impact on lysosomal iron release (lysosomotropic action).

In summary, moderate iron overload caused no basal cardiac dysfunction, but did have detrimental consequences to I/R hearts. Reperfusion time course data implicate the participation of at least two pools of redox-active iron in the injury process, the cytosolic iron pool liberated at the onset of reperfusion, and the larger second wave of iron release at later times of reperfusion, which is more exaggerated in the iron-loaded heart versus the control heart, and may originate from severely destabilized lysosomes. D-Pro significantly reduced reperfusion injury to iron-loaded I/R rat hearts, and its membrane-stabilizing antioxidant and lysosomotropic properties may underlie its cardioprotection, independent of β-receptor blockade.

The authors thank Mr. Jonathan Hall and Ms. Armida Torres-Duarte for their excellent technical assistance on this project.

1. Giardina PJ, Grady RW. Chelation therapy in beta-thalassemia: the benefits and limitations of desferrioxamine. Semin Hematol 32:304–312, 1995.
2. Hershko C, Link G, Cabantchik I. Pathophysiology of iron overload. Ann N Y Acad Sci 850:191–201, 1998.
3. Masini A, Trenti T, Ceccarelli D, Muscatello U. Mitochondrial involvement in causing cell injury in experimental hepatic iron overload. Ann N Y Acad Sci 488:517–519, 1986.
4. Gunther T, Hollriegl V, Vormann J, Disch G, Classen HG. Effects of Fe loading on vitamin E and malondialdehyde of liver, heart and kidney from rats fed diets containing various amounts of magnesium and vitamin E. Magnesium-Bulletin 14:88–93, 1992.
5. Pepper JR, Mumby S, Gutteridge JM. Blood cardioplegia increases plasma iron overload and thiol levels during cardiopulmonary bypass. Ann Thorac Surg 60:1735–1740, 1995.
6. Mariotti E, Angelucci E, Agostini A, Baronciani D, Sgarbi E, Lucarelli G. Evaluation of cardiac status in iron-loaded thalassaemia patients following bone marrow transplantation: improvement in cardiac function during reduction in body iron burden. Br J Haematol 103: 916–921, 1998.
7. Jensen PD, Olsen N, Bagger JP, Jensen FT, Christensen T, Ellegaard J. Cardiac function during iron chelation therapy in adult non-thalassaemic patients with transfusional iron overload. Eur J Haematol 59:221–230, 1997.
8. Lombardo T, Tamburino C, Bartoloni G, Morrone ML, Frontini V, Italia F, Cordaro S, Privitera A, Calvi V. Cardiac iron overload in thalassemic patients: an endomyocardial biopsy study. Ann Hematol 71:135–141, 1995.
9. Politi A, Sticca M, Galli M. Reversal of haemochromatotic cardiomyopathy in beta thalassaemia by chelation therapy. Br Heart J 73:486–487, 1995.
10. Kremastinos DT, Tiniakos G, Theodorakis GN, Katritsis DG, Toutouzas PK. Myocarditis in beta-thalassemia major. A cause of heart failure. Circ 91:66–71, 1995.
11. Whittaker P, Hines FA, Robl MG, Dunkel VC. Histopathological evaluation of liver, pancreas, spleen, and heart from iron-overloaded Sprague-Dawley rats. Toxicol Pathol 24:558–563, 1996.
12. Brun A, Brunk U. Histochemical indications for lysosomal localization of heavy metals in normal rat brain and liver. J Histochem Cytochem 18:820–827, 1970.
13. van der Schouw YT, van der Veeken PMWC, Kok FJ, Koster JF,

Schouten EG, Hofman A. Iron status in the acute phase and six weeks after myocardial infarction. Free Rad Biol Med 8:47–53, 1990.

14. Aust SD, White BC. Iron chelation prevents tissue injury following ischemia. Free Rad Biol Med 1:1–17, 1985.
15. van der Kraaij AMM, Mostert L, van Eijk HG, Koster JF. Iron-load increases the susceptibility of rat hearts to oxygen reperfusion damage. Protection by the antioxidant (+)-cyanidanol-3 and deferoxamine. Circ 78:442–449, 1988.
16. Voogd A, Sluiter W, van Eijk HG, Koster JF. Low molecular weight iron and the oxygen paradox in isolated rat hearts. J Clin Invest 90: 2050–2055, 1992.
17. Funk F, Lenders JP, Crichton RR, Schneider W. Reductive mobilisation of ferritin iron. Eur J Biochem 152:167–172, 1985.
18. Biemond P, van Eijk HG, Swaak AJ, Koster JF. Iron mobilization from ferritin by superoxide derived from stimulated polymorphonuclear leukocytes. Possible mechanism in inflammation diseases. J Clin Invest 73:1576-1579, 1984.
19. Prasad MR, Engelman RM, Jones RM, Das DK. Effect of oxyradicals on oxymyoglobin. Deoxygenation, haem removal and iron release. Biochem J 263:731–736, 1989.
20. Koster JF, Voogd A, Ruigrok TJ, Sluiter W. Depletion of preischemic glycogen reduces the increase of low molecular weight iron during ischemia. Ann N Y Acad Sci 793:74–84, 1996.
21. Hershko C, Link G, Cabantchik I. Pathophysiology of iron overload. Ann N Y Acad Sci 850:191–201, 1998.
22. Eaton JW, Qian M. Molecular bases of cellular iron toxicity. Free Rad Biol Med 32:833–840, 2002.
23. Whittaker P, Wamer WG, Chanderbhan RF, Dunkel VC. Effects of alpha-tocopherol and beta-carotene on hepatic lipid peroxidation and blood lipids in rats with dietary iron overload. Nutr Cancer 25:119–128, 1996.
24. Bartfay WJ, Hou D, Brittenham GM, Bartfay E, Sole MJ, Lehotay D, Liu PP. The synergistic effects of vitamin E and selenium in iron-overloaded mouse hearts. Can J Cardiol 14:937–941, 1998.
25. Mak IT, Weglicki WB. Protection by beta-blocking agents against free radical-mediated sarcolemmal lipid peroxidation. Circ Res 63:262–266, 1988.
26. Mak IT, Arroyo CM, Weglicki WB. Inhibition of sarcolemmal carbon-centered free radical formation by Propranolol. Circ Res 65:1151–1156, 1989.
27. Mak IT, Kramer JH, Freedman AM, Tse SYH, Weglicki WB. Oxygen free radical-mediated injury of myocytes-protection by Pro. J Mol Cell Cardiol 22:687–695, 1990.
28. Weglicki WB, Mak IT, Dickens BF, Kramer JH. Beta-blockers, calcium channel blockers and the sulfhydryl-ACE inhibitors demonstrate protection against free radical-mediated injury of cardiovascular cells and membranes. Rev Port Cardiol 11:1009–1011, 1992.
29. Kramer JH, Mak IT, Freedman AM, Weglicki WB. Propranolol reduces anoxia/reoxygenation-mediated injury of adult myocytes through an anti-radical mechanism. J Mol Cell Cardiol 23:1231–1244, 1991.
30. Kramer JH, Misik V, Weglicki WB. Lipid peroxidation-derived free radical production and post-ischemic myocardial reperfusion injury. Ann N Y Acad Sci 723:180–196, 1994.
31. Cramb G. Selective lysosomal uptake and accumulation of the beta-adrenergic antagonist propranolol in cultured and isolated cell systems. Biochem Pharmacol 35:1365–1372, 1986.
32. Yu Z, Persson HL, Eaton JW, Brunk UT. Intralysosomal iron: a major determinant of oxidant-induced cell death. Free Rad Biol Med 34: 1243–1252, 2003.
33. Yang T, Brittenham GM, Dong WQ, Levy MN, Obejero-Paz CA, Kuryshev YA, Brown AM. Deferoxamine prevents cardiac hypertrophy and failure in the gerbil model of iron-induced cardiomyopathy. J Lab Clin Med 142:332–340, 2003.
34. Oudit GY, Trivieri MG, Khaper N, Husain T, Wilson GJ, Liu P, Sole MJ, Backx PH. Taurine supplementation reduces oxidative stress and improves cardiovascular function in an iron-overload murine model. Circ 109:1877–1885, 2004.
35. Obejero-Paz CA, Yang T, Dong WQ, Levy MN, Brittenham GM, Kuryshev YA, Brown AM. Deferoxamine promotes survival and prevents electrocardiographic abnormalities in the gerbil model of iron-overload cardiomyopathy. J Lab Clin Med 141:121–130, 2003.
36. Bartfay WJ, Bartfay E. Iron-overload cardiomyopathy: evidence for a free radical–mediated mechanism of injury and dysfunction in a murine model. Biol Res Nurs 2:49–59, 2000.
37. Kramer JH, Misik V, Weglicki WB. Magnesium-deficiency potentiates free radical production associated with post-ischemic injury to rat hearts: vitamin E affords protection. Free Rad Biol Med 16:713–723, 1994.
38. Kramer JH, Arroyo CM, Dickens BF, Weglicki WB. Spin-trapping evidence that graded myocardial ischemia alters post-ischemic superoxide production. Free Radical Biol Med 3:153–159, 1987.
39. Mak IT, Misra HP, Weglicki WB. Temporal Relationship of free radical-induced lipid peroxidation and loss of latent enzyme activity in highly enriched hepatic lysosomes. J Biol Chem 258:13733–13737, 1983.
40. Dickens BF, Mak IT, Weglicki WB. Lysosomal lipolytic enzymes, lipid peroxidation, and injury. Mol Cell Biochem 82:119–123, 1988.
41. Murthi SB, Kramer JH, Wise RM, Mak IT, Chmielinska J, Weglicki WB. Chronic D-Pro treatment reduces iron-overload-mediated systemic injury and loss of rat heart function (abstract). FASEB J 17:A1163, 2003.
42. Pucheu S, Coudray C, Tresallet N, Favier A, de Leiris J. Effect of iron overload in the isolated ischemic and reperfused rat heart. Cardiovasc Drugs Ther 7:701–711, 1993.
43. Coudray C, Pucheu S, Boucher F, Arnaud J, de Leiris J, Favier A. Effect of ischemia/reperfusion sequence on cytosolic iron status and its release in the coronary effluent in isolated rat hearts. Biol Trace Elem Res 41:69–75, 1994.
44. Kramer JH, Lightfoot F, Weglicki WB. Cardiac tissue iron: effects on postischemic function and free radical production, and its possible role during preconditioning. Cell Mol Biol 46:1313–1327, 2000.
45. Chevion M, Liang Y, Har-El R, Berenshtein E, Uretzky G, Kitrossky N. Copper and iron are mobilized following myocardial ischemia: possible predictive criteria for tissue injury. Proc Natl Acad Sci U S A 90:1102–1106, 1993.
46. Opie LH. Myocardial ischemia—metabolic pathways and implications of increased glycolysis. Cardiovasc Drugs Ther 4:777–790, 1990.
47. Schaffer SW, Safer B, Ford C, Illingworth J, Williamson JR. Respiratory acidosis and its reversibility in perfused rat heart: regulation of citric acid cycle activity. Am J Physiol 234:H40–H51, 1978.
48. Levine RL. Ischemia: from acidosis to oxidation. FASEB J 7:1242–1246, 1993.
49. Decker RS, Poole AR, Griffin EE, Dingle JT, Wildenthal K. Altered distribution of lysosomal cathepsin D in ischemic myocardium. J Clin Invest 59:911–921, 1977.
50. Mair J. Tissue release of cardiac markers: from physiology to clinical applications. Clin Chem Lab Med 37:1077–1084, 1999.
51. Lesnefsky EJ. Tissue iron overload and mechanisms of iron-catalyzed oxidative injury. Adv Exp Med Biol 366:129–146, 1994.
52. Boucher F, Pucheu S, Coudray C, Favier A, de Leiris J. Evidence of cytosolic iron release during post-ischaemic reperfusion of isolated rat hearts: influence on spin-trapping experiments with DMPO. FEBS Lett 302:261–264, 1992.
53. Arroyo CM, Kramer JH, Dickens BF, Weglicki WB. Identification of free radicals in myocardial ischemia/ reperfusion by spin trapping with nitrone DMPO. FEBS Lett 221:101–104, 1987.
54. Arroyo CM, Kramer JH, Leiboff RH, Mergner GW, Dickens BF, Weglicki WB. Spin trapping of oxygen and carbon-centered free

radicals in ischemic canine myocardium. Free Rad Biol Med 3:313–316, 1987.

55. Weglicki WB, Arroyo CM, Kramer JH, Mak IT, Leiboff RH, Mergner GW, Dickens BF. Applications of spin trapping/ESR techniques in models of cardiovascular injury. In: Cerutti PA, Fridovich I, McCord JM, Eds. Oxy-Radicals in Molecular Biology and Pathology. New York: Alan R Liss, pp357–364, 1988.
56. Cobbe SM, Poole-Wilson PA. The time of onset and severity of acidosis in myocardial ischaemia. J Mol Cell Cardiol 12:745–760, 1980.
57. Weglicki WB, Mak IT, Dickens BF, Stafford RE, Komarov AM, Gibson B, Cassidy MM, Phillips TM, Kramer JH. Neuropeptides, free radical stress and antioxidants in models of Mg-deficient cardiomyopathy. In: Theophanides T, Anastassopoulou J, Eds. Magnesium: Current Status and New Developments. Dordrecht, the Netherlands: Kluwer Academic Publishers, pp169–178, 1997.
58. Dickens BF, Weglicki WB, Boehme PA, Mak IT. Antioxidant and lysosomotropic properties of acridine Propranolol: protection against oxidative endothelial cell injury. J Mol Cell Cardiol 34:129–137, 2002.