Low-intensity exercise training during doxorubicin treatment protects against cardiotoxicity

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Chicco, Adam J., David S. Hydock, Carole M. Schneider, and Reid Hayward. Low-intensity exercise training during doxorubicin treatment protects against cardiotoxicity. J Appl Physiol 100: 519–527, 2006. First published October 6, 2005; doi:10.1152/japplphysiol.00148.2005.—Doxorubicin (Dox) is a highly effective antineoplastic antibiotic associated with a dose-limiting cardiotoxicity that may result in irreversible cardiomyopathy and heart failure. The purpose of this study was to examine the effects of low-intensity exercise training (LIET) during the course of Dox treatment on cardiac function, myosin heavy chain expression, oxidative stress, and apoptosis activation following treatment. Male Sprague-Dawley rats either remained sedentary or were exercise trained on a motorized treadmill at 15 m/min, 20 min/day, 5 days/wk (Monday through Friday) for 2 wk. During the same 2-wk period, Dox (2.5 mg/kg) or saline was administered intraperitoneally to sedentary and exercised rats 3 days/wk (Monday, Wednesday, Friday) 1–2 h following the exercise training sessions (cumulative Dox dose: 15 mg/kg). Five days following the final injections, hearts were isolated for determination of left ventricular (LV) function, lipid peroxidation, antioxidant enzyme protein expression, 72-kDa heat shock protein expression, caspase-3 activity, and myosin heavy chain isoform expression. Dox treatment significantly impaired LV function and increased caspase-3 activity in sedentary animals (P < 0.05). LIET attenuated the LV dysfunction and apoptotic signal activation induced by Dox treatment and increased glutathione peroxidase expression, but it had no significant effect on lipid peroxidation, protein expression of myosin heavy chain isoforms, 72-kDa heat shock protein, or superoxide dismutase isoforms. In conclusion, our data suggest that LIET applied during chronic Dox treatment protects against cardiac dysfunction following treatment, possibly by enhancing antioxidant defenses and inhibiting apoptosis.

adressamycin; apoptosis; physical activity; cardioprotection; myosin heavy chain

DOXORUBICIN (DOX) IS AN ANTINEOPLASTIC antibiotic widely used in the treatment of a variety of cancers. Unfortunately, the clinical use of this highly effective anticancer drug is limited due to a severe, dose-dependent cardiotoxicity characterized by acute cardiac injury that may progress to irreversible cardiomyopathy and congestive heart failure months to years following treatment (22, 26). Early signs of cardiac dysfunction following Dox treatment have been found to be highly predictive of future heart failure in human (26) and animal studies (25), and substantial effort has been devoted to developing strategies for avoiding acute Dox cardiotoxicity while maintaining the drug’s antineoplastic effectiveness. Of the several molecular mechanisms of cardiotoxicity that have been postulated (see Ref. 39 for review), generation of reactive oxygen species (ROS) (13, 25, 34, 38) and induction of apoptosis (4, 17) are believed to play central roles. Prophylactic treatment before and during Dox treatment has proved to be only partially effective in the clinical setting, and the therapeutic value of Dox remains limited by its cardiotoxicity.

Exercise training has been repeatedly shown to confer protection against a variety of acute and chronic myocardial insults, such as ischemia and reperfusion (see Ref. 29 for review). Recent evidence from our laboratory (9) and others (3, 4) indicates that hearts from physically active rats exhibit resistance against the cardiac dysfunction, lipid peroxidation, and apoptosis induced by acute Dox exposure. Whereas these studies suggest that long-term exercise training protects against acute Dox cardiotoxicity, the benefits of initiating an exercise training protocol during a chronic Dox treatment regimen have not been previously examined. The purpose of the current investigation was to determine whether low-intensity exercise training (LIET) initiated during a 2-wk course of Dox treatment confers protection against cardiac dysfunction and apoptosis activation following the chronic Dox treatment regimen. Given their putative roles in mediating exercise-induced cardioprotection, the effects of LIET and Dox treatment on the myocardial protein expression of superoxide dismutase (SOD) isoforms [manganese (Mn-SOD) and copper and zinc SOD (CuZn-SOD)], glutathione peroxidase (GPX), and the inducible isoform of 70-kDa heat shock protein family (HSP72) were examined. In addition, given prior evidence for an effect of chronic Dox exposure on cardiac myosin heavy chain (MHC) isoform composition (11), the effect of Dox and LIET on the relative contents of the α- and β-MHC isoforms was also evaluated.

MATERIALS AND METHODS

Animal subjects and experimental design. All experimental procedures were approved by the University of Northern Colorado Institutional Animal Care and Use Committee and were in compliance with guidelines established by the American Physiological Society. Male Sprague-Dawley rats (175–200 g) were randomized into one of four experimental groups: sedentary (Con; n = 6), sedentary during Dox treatment (Dox; n = 8), exercise trained (Ex; n = 6), or exercise trained during Dox treatment (Dox+Ex; n = 8). A summary of the experimental protocol is illustrated in Fig. 1. Rats were housed in a temperature-controlled animal facility maintained on a 12:12-h light-dark cycle with chow and water provided ad libitum. All rats were acclimated to the treadmill (8–10 m/min, 5 min/day) and handling

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EXERCISE DURING DOxorubicin TREATMENT

Myocardial lipid peroxidation. To examine the effect of Dox and LIET on myocardial lipid peroxidation, a common manifestation of cellular injury mediated by ROS, malondialdehyde and 4-hydroxy-alkenals (MDA + 4-HAE) were analyzed in LV tissue following the isolated heart functional assessments described above with the use of a spectrophotometric assay kit (Calbiochem, San Diego, CA). Frozen LV tissue was homogenized in ice-cold 20 mM Tris-HCl, pH 7.4, containing 5 mM butylated hydroxytoluene (to prevent lipid peroxidation during homogenization) at a dilution of 1:5 (wt/vol) and centrifuged at 3,000 g for 10 min at 4°C. The supernatant was collected and used for analyses. MDA + 4-HAE content is expressed relative to the protein content of sample supernatant determined by the method of Bradford using bovine serum albumin as the standard.

Western immunoblotting. To examine the effect of LIET on putative candidates of cardioprotection, GPX, CuZn-SOD, Mn-SOD, and HSF72 protein expressions were determined in LV samples obtained from all hearts by using Western immunoblotting methods previously described (23). Briefly, LV samples were homogenized in a cold homogenizing buffer containing (in mM) 100 KCl, 5 MOPS, 5 MgCl₂, 1 ATP, 1 EGTA (pH 7.4), and a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Homogenates were then diluted 1:3 (vol/vol) in lysis buffer containing 20 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, and 0.1% SDS (pH 7.4), allowed to incubate at room temperature for 10 min, and then centrifuged at 3,000 g for 5 min. The supernatant was then assayed for total protein (7), and a volume of supernatant containing 50 μg (for GPX and HSF72 assessments) or 3 μg (for SOD isoform assessments) of LV protein was electrophoresed on polyacrylamide gels for separation of proteins by molecular weight. Proteins were then transferred to membranes (Amersham, Piscataway, NJ); Ponceau stained to confirm uniform sample loading and transfer; and then washed and incubated with an alkaline phosphatase-conjugated polyclonal antibody specific for rat HSP72, or polyclonal antibodies specific for CuZn-SOD, Mn-SOD (Stressgen, Victoria, BC, Canada), or GPX (Abcam, Cambridge, MA). HSF72 blots were then reacted with 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium substrate (Sigma Chemical, St Louis, MO) and scanned for band density analysis. GPX and SOD isoform blots were incubated with a secondary horseradish peroxidase-conjugated goat anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) followed by a chemiluminescent substrate (Western Lightning, Perkin-Elmer Life Sciences, Boston, MA) and developed on film. Quantification of protein band densities were performed on computerized scans of immunoblots using ImageJ densitometry software (NIH, Bethesda, MD).

Caspase-3 activity assay. To examine the effects of Dox treatment and LIET on the initiation of apoptosis, LV tissue was homogenized in the lysis buffer described above and incubated in 50 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, and 20% glycerol containing 10 mM fluorogenic caspase-3 substrate (Ac-DEVD-AMC, Calbiochem) in a Fluoroscan Ascent fluorometric plate reader (Thermo, Milford, MA) for 90 min at room temperature. Fluorescence was monitored at 360/460 nm at 2-min intervals, and caspase-3 activity was expressed as relative fluorometric units per milligram of sample protein.

MHC isoform analyses. LV tissues were homogenized in 250 mM sucrose, 100 mM KCl, 5 mM EDTA, and 20 mM Tris base, pH 6.8, using a glass tissue homogenizer, sonicated, and centrifuged for 10 min at 1,000 g (4°C). The pellet was resuspended in a washing buffer (175 mM KCl, 0.5% Triton X-100, 2 mM EDTA, 20 mM Tris base, pH 6.8, and centrifuged again for 10 min at 1,000 g (4°C). The pellet was then resuspended in the washing buffer a second time and centrifuged for 10 min at 1,000 g (4°C), and then it was resuspended in a final resuspension buffer (150 mM KCl and 20 mM Tris base, pH 7.0). Total protein was determined according to Bradford (7), and protein concentration was adjusted to 6 mg/ml with the final resuspension buffer. Samples were then diluted to 0.125 mg/ml with 2×...
Laemmli sample buffer (20% glycerol, 16% 1 M Tris, pH 6.8, 4% SDS, 1% β-mercaptoethanol, and 0.2% bromphenol blue). Samples were boiled for 2 min and placed on ice for 10 min before being loaded onto polyacrylamide gels. MHC isoforms were separated via SDS-PAGE using stacking gels consisting of 4% acrylamide-bis and separating gels consisting of 8% acrylamide-bis. Samples (0.94-μg protein) were loaded onto gels and electrophoresed at 100 V until tracking dye reached the bottom of the gels. Gels were stained with Coomassie blue. Gels were scanned and digitized using UN-SCAN IT Gel Digitizing Software (Orem, UT).

Statistical analysis. All data are expressed as means ± SE. Differences among the four experimental groups were assessed by using a two-way (Ex × Dox) ANOVA with independent sample t-tests post hoc when appropriate to examine between-group differences. P values ≤0.05 were considered statistically significant.

RESULTS

General observations. All animals in the Ex and Ex+Dox groups were compliant with the training protocol and required only occasional light prodding to maintain the treadmill exercise. Dox treatment led to mild lethargy and abdominal fluid accumulation (ascites) at the time of death, with no discernable differences observed between the Dox and Dox+Ex groups. The effect of exercise training and Dox treatment on heart and body weight is presented in Table 1. As expected for young developing rats, animals in the Con and Ex group gained 70 g of body weight during the 2-wk experimental period. The 2-wk LIET protocol did not elicit any statistically significant differences in body weight, heart weight, or cardiac function among the four groups. GPX protein was significantly increased in the Ex group (P < 0.05 vs. Con) but not in the Dox+Ex group. ANOVA analyses indicated a slight depressive effect of Dox treatment on GPX protein expression (P = 0.056). HSP72 protein expression was significantly higher in the Dox-treated animals compared with all other groups (51% higher than Con; P = 0.05), indicating that Dox treatment led to HSP72 induction. This effect was completely abolished by LIET during Dox treatment.

Caspase activity. The effects of LIET and Dox on caspase-3 activity are presented in Fig. 5. Dox treatment led to a 14% increase in caspase-3 activity in the LV compared with Con (P < 0.05), suggesting that Dox activated the caspase-3 apoptotic signaling pathway. ANOVA analyses indicated a significant suppressive effect of LIET on caspase-3 activity (P < 0.01). LIET during Dox treatment abolished the Dox-induced increase in caspase activity and reduced activity to ~20% below Con levels (P < 0.05), suggesting that LIET attenuates myocardial apoptotic signaling in the presence and absence of Dox treatment.

Effect of Dox and LIET on cardiac MHC isoforms. The effects of LIET and Dox on cardiac MHC isoform distribution are presented in Fig. 6. There was a slight trend toward an increase in the relative content of the β- (slow) MHC isoforms in the Dox-treated animals (effect of Dox, P = 0.24), but no statistically significant changes in MHC isoforms were detected following the LIET or Dox treatment protocols.

DISCUSSION

In this study, we have provided novel evidence of cardioprotection against Dox-induced cardiac dysfunction by LIET.

### Table 1. Effect of exercise training and doxorubicin treatment on animal characteristics

<table>
<thead>
<tr>
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<th>Con</th>
<th>Ex</th>
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<tr>
<td>n</td>
<td>6</td>
<td>6</td>
<td>8</td>
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<td>Heart/body weight, mg/g</td>
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<td>3.95±0.05</td>
<td>3.70±0.07</td>
<td>3.68±0.06</td>
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Values are means ± SE; n, no. of animals. Con, sedentary control; Ex, exercise trained; Dox, doxorubicin; Dox + Ex, exercise trained during doxorubicin treatment; Δ, change. *Significantly less than Con and Ex, P < 0.05.
initiated during a 2-wk course of Dox treatment. Interestingly, this effect was observed in the absence of any LIET-induced increase in the protein expression of SOD isoforms or HSP72, putative mediators of exercise-induced cardioprotection against Dox cardiotoxicity (4), indicating that induction of these proteins is not required for cardioprotection against acute Dox-induced cardiac dysfunction. In contrast, LIET during Dox treatment attenuated the Dox-induced increase in caspase-3 activity, providing the first evidence that exercise may be cardioprotective in this model by inhibiting apoptotic signaling. Our data confirm prior evidence that Dox treatment arrests body and heart weight gain in young animals. These effects were not modified by LIET during treatment and are most likely explained by previous reports of a Dox-mediated inhibition of cardiomyocyte protein synthesis and a decrease in caloric intake during Dox treatment (6, 15, 36).

Effects of chronic Dox treatment. The 2-wk Dox treatment regimen used in the present study resulted in a significant impairment of LV function 5 days following treatment, which is in agreement with previous investigations that utilized a similar Dox administration schedule (27, 34). The mechanisms of Dox-induced cardiac dysfunction are believed to be distinct from the drug’s antineoplastic effects and have been suggested to involve the production of ROS (39) and cardiomyocyte apoptosis activation (4, 17). Dox is capable of redox cycling with oxidoreductases in cardiomyocytes (e.g., NADH dehydrogenase) to generate the superoxide radical, leading to an increase in intracellular ROS (12). The resulting oxidative stress has been linked to a myriad of destructive processes, such as membrane lipid peroxidation, apoptosis, and injury to DNA (10, 13, 25).

Impairment of the myocardial contractile function and relaxation has been associated with a shift in the ventricular MHC isoform distribution from the “fast” α-MHC isoform to the “slow” β-MHC isoform following chronic Dox treatment in vitro (11). In the present study, there was a slight trend toward a MHC shift to the β-isoform, but no significant alteration of the MHC isoform distribution was detected following chronic Dox treatment in vivo. While it is reasonable to

Fig. 2. Effect of exercise training and Dox on cardiac function in hearts isolated from Con (n = 6), Dox (n = 8), Ex (n = 6), and Dox + Ex (n = 8) animals. Left ventricular developed pressure (LVDP), rate of LV pressure development (+dP/dt), rate of LV relaxation (−dP/dt), and coronary flow rate per gram of heart weight were recorded following equilibration under paced conditions (300 beats/min) following a 15-min equilibration period. Values are means ± SE. *P < 0.05 vs. all other groups. †P < 0.05 vs. Dox.
speculate that a more pronounced shift toward the β-MHC isoform may have occurred in the Dox-treated animals over a longer treatment protocol, our data suggest that alterations in cardiac MHC isoforms are not responsible for the acute cardiac dysfunction observed following chronic Dox treatment.

An interesting finding in the present study that may provide further insight into the effects of Dox on the myocardium was an increase in HSP72 protein expression in the LV of the sedentary Dox-treated animals. Myocardial HSP72 induction is believed to occur as an adaptive response to cellular stress and has been previously reported following a variety of cardiac insults. HSP72 induction has been observed in isolated cardiomyocytes following Dox exposure in vitro (14); however, to our knowledge, the present study provides the first evidence of a sustained increase in myocardial HSP72 protein for up to 5 days following chronic Dox treatment in vivo. While the molecular mechanism of this effect was not determined in the present study, induction of HSPs of the 70-kDa family has been observed following a wide variety of cellular stresses, including increased intracellular calcium (37), inhibition of mitochondrial respiration (5), and exposure to ROS (19), all of which have been associated with Dox treatment (2, 12, 31).

Cardioprotective effects of LIET during Dox treatment. Previous studies from our laboratory (9) and others (3, 4, 18) suggest that physically active animals resist the functional and biochemical manifestations of Dox cardiotoxicity. However, before the present study, the effect of ET applied during Dox therapy in previously untrained animals had not been examined. Our data indicate that LIET initiated during chronic Dox treatment protects against the LV dysfunction observed in the sedentary Dox-treated rats. Interestingly, LIET preserved LV function, despite having no effect on the growth-arresting effects of Dox on the heart. This suggests that LIET did not preserve LV function simply by maintaining the cardiac growth curve and that the acute Dox-induced LV dysfunction observed in the sedentary animals is not due solely to impaired cardiac growth.

To explore possible mechanisms of the LIET-induced preservation of cardiac function, myocardial antioxidant enzymes, HSP72, apoptotic signaling, lipid peroxidation, and MHC isoform composition were examined. Exercise-induced cardioprotection is often associated with training-induced increases in myocardial SOD isoforms and HSP72 content (28, 30), which have been proposed to mediate exercise-induced cardioprotection by providing resistance against the superoxide radical and preserving protein function during states of cellular stress, respectively. Elevations in myocardial levels of SOD or HSP72 have been shown to confer cardioprotection against Dox (16, 38) and have recently been associated with exercise-induced cardioprotection against Dox (4). In contrast to these studies, the trained animals in the present study did not exhibit a significant increase in myocardial HSP72 or SOD isoform contents relative to sedentary animals. A plausible explanation for this finding is that the LIET protocol did not provide a sufficiently intense stimulus for HSP72 and SOD induction in the heart. Increases in myocardial SOD and HSP72 content have been observed following short-term ET lasting <1 to >20 wk when intensity was very high (≥30 m/min, ≥10% grade) (8, 28), but they were not observed following ET at lower intensities (e.g., <24 m/min) (20, 24, 28). LIET was selected in the present study, rather than a higher intensity running protocol, in an effort to simulate the daily low-intensity activity reported to be safe and beneficial in individuals undergoing cancer chemotherapy (32, 33). While it is possible that a higher intensity ET regimen may have evoked increases in SOD and HSP and provided additional protection against Dox cardiotoxicity, it is unlikely that such a protocol would be practical in previously untrained cancer patients undergoing chemotherapy in the clinical setting.

Interestingly, the present study is not the first investigation to demonstrate exercise-induced cardioprotection in the absence of increased myocardial SOD (28) or HSP72 expression (21, 35), indicating that other mechanisms of exercise-induced cardioprotection must exist. Myocardial GPX protein expression was elevated by LIET in the saline-treated animals, but not in Dox-treated animals, suggesting that Dox treatment prevented the training-induced GPX induction. Kanter et al. (18) reported a decrease in cardiac GPX protein following chronic Dox treatment in sedentary male mice, but this was attenuated by swim training before and during the chronic Dox treatment protocol. These discrepant findings may be explained by the fact that the swim-training regimen used by Kanter et al. was substantially more intense than the LIET treadmill protocol used in the present study and may have, therefore, provided a stronger stimulus for cardiac GPX expression.

Accumulating evidence supports the role of cardiomyocyte apoptosis in the pathogenesis of Dox cardiomyopathy (1, 17). Dox treatment in the present study resulted in an increase in cardiac caspase-3 activity in sedentary animals, but not in Dox+Ex animals, suggesting that LIET during Dox treatment abolished the Dox-induced activation of apoptotic signaling in the heart. Inhibition of the apoptotic pathways by exercise training has been recently reported by others (4), yet the...
mechanism of this Dox-induced increase in caspase activity and its inhibition by LIET are not immediately clear from this study. A Dox-mediated increase in ROS has been implicated in the apoptosis induction reported in previous studies (17), and myocardial lipid peroxidation may shed some light on this possibility. In our studies, we observed no differences in MDA/H110014-HAE between any of the experiments groups. However, it should be noted that our lipid peroxidation measurements were made 5 days after the termination of Dox treatment and LIET. As a result, we are unaware of the degree of oxidative stress at the time Dox treatment and LIET were terminated. It is possible that differences in MDA + 4-HAE may have been present during or immediately after Dox treatment and LIET, and these differences may have waned during the subsequent 5 days.

Further insight into the cardioprotection afforded by LIET may come from our evidence that LIET prevented the Dox-induced increase in HSP72 protein observed in the sedentary rats. As mentioned, HSP72 induction has been observed following a wide variety of cellular stresses, many of which have been previously associated with Dox cardiotoxicity. The fact that LIET mitigated Dox-induced HSP72 expression suggests that this type of physical activity may attenuate one, or several, of these cellular stresses necessary for HSP72 upregulation. Further examination of the effects of Dox, LIET, and their interaction on myocardial HSP72 transcription pathways may shed further light on the subcellular effects of Dox in cardiomyocytes and the mechanisms by which LIET during Dox treatment protects against cardiotoxicity.

In summary, the results of this study provide novel evidence of a cardioprotective effect of LIET applied during the course of Dox treatment in previously untrained animals. The LIET-induced preservation of cardiac function coincided with a prevention of Dox-induced activation of caspase-3 apoptotic signaling but not with an increase in myocardial antioxidant defenses or HSP72 protein. Our data also indicate that Dox can induce significant cardiac dysfunction in the absence of any increase in myocardial lipid peroxidation or alterations in the myocardial MHC isoform composition.

**Limitations.** While the present study provides important information about an exercise-induced protection against the cardiotoxic effects of Dox treatment, the effect of exercise...
training during Dox chemotherapy on the antineoplastic effects of Dox cannot be determined by this study. Furthermore, it is important to note that, whereas previous evidence suggests a strong association between the acute and chronic manifestations of Dox cardiotoxicity (25, 26), our finding that LIET protects against acute Dox dysfunction does not necessarily imply protection against the delayed forms of Dox cardiomyopathy. In particular, the existence of a greatly delayed Dox cardiomyopathy becoming evident as long as 20 yr after Dox treatment for childhood cancer has been suggested to result from a Dox-mediated impairment of cardiac growth and development (22). The LIET protocol used in the present study did not prevent the growth-arresting effects of Dox on the heart, suggesting that LIET may not provide protection against this type of delayed Dox cardiomyopathy. Therefore, further study should be conducted to examine whether exercise affords similar protection against any long-term effects of Dox treatment on the heart.

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