Erythropoietin protects against doxorubicin-induced heart failure

Hania Ibrahim Ammar,1 Soliman Saba,1 Rasha Ibrahim Ammar,1 Laila Ahmed Elsayed,1 Wael Botros Abu-Alyamin Ghaly,1 and Sanjiv Dhingra2

1Department of Physiology, Faculty of Medicine, Cairo University, Cairo, Egypt; and 2Toronto General Research Institute, University Health Network, Toronto, Canada

Submitted 1 November 2010; accepted in final form 29 September 2011


The antineoplastic drug doxorubicin is effective in the treatment of a broad spectrum of malignancies, but its clinical use is limited by adverse side effects: irreversible degenerative cardiomyopathy and congestive heart failure (30, 33). The efficacy of doxorubicin [adriamycin (AD)] as a cytotoxic agent for the treatment of various human tumors prompted a search for treatments to reduce or prevent doxorubicin-induced cardiomyopathy and congestive heart failure (24). So far, however, available treatments to protect the heart from doxorubicin-induced damage have been varied and limited.

The cytokine erythropoietin (EPO) is produced by the kidney and is indispensable for the proliferation, survival, and differentiation of erythroid progenitor cells (39). EPO receptors have also been identified in nonhematopoietic tissues, including the heart (34, 38). Recent studies (7, 27, 31) suggest that EPO also exerts a cardioprotective effect against infarction and ischemia-reperfusion injury. EPO administration before or during ischemia significantly enhanced left ventricular (LV) contractility and the recovery of cardiac function after myocardial ischemia and reperfusion injury. Several studies (8, 18) suggested that EPO treatment improved ventricular contractile function in animal models of heart failure and that the benefit was associated with reduced cardiomyocyte apoptosis. Transgenic mice deficient in the EPO receptor showed a more rapid progression of heart failure associated with a significant reduction in capillary density (2, 9). In the clinical setting, Namiuchi et al. (28) reported that a higher serum level of EPO predicted a smaller infarct size in patients following a myocardial infarction. Furthermore, EPO treatment increased the exercise capacity in patients with moderate to severe chronic heart failure (23).

With this background, the present study was designed to determine the protective role of EPO in doxorubicin-induced heart failure and its detailed mechanism.

MATERIALS AND METHODS

In Vivo Studies

Animals. Male Wistar rats weighing 150 ± 10 g were used in this study. Animals were kept in the animal care facility of Cairo University and housed in the chip-bedded cages under a 12:12-h light-dark cycle. The rats were provided with ordinary rat chow and water ad libitum. The experimental protocol and procedures were approved by the Institutional Animal Care and Use Committee of Cairo University.

Experimental design. Animals were randomly allocated into the following groups: the control group (n = 10) received saline (0.2 ml ip, vehicle solution) three times per week for 4 wk, and the EPO group (n = 10) received EPO (SEDICO Pharmaceutical Egypt; 1,000 IU/kg body wt sc) three times per week for 4 wk. This dose of EPO was effective in preventing cardiac remodeling after coronary ligation (29). AD group (n = 10) rats were injected with doxorubicin (AD; PHARMACIA Italia; 2.5 mg/kg body wt ip) in six equal doses over the period of 2 wk (16). The EPO-AD group (n = 10) received both EPO (1,000 IU/kg body wt sc) and doxorubicin (2.5 mg/kg body wt ip). AD was stopped after 2 wk and EPO treatment was continued for 4 wk (16).

Noninvasive blood pressure measurements and echocardiography were performed for all rats at the beginning and at the end of the experimental period (4 wk). Blood samples were collected for the measurement of hematocrit (HCT) at the beginning and at the end of experimental period.

At the end of the study, hearts from all treatments groups were excised for ischemia-reperfusion studies described below. The hearts were then placed in phosphate-buffered formalin for immunohistochemical analysis for angiogenesis (CD31 expression).

Arterial blood pressure measurements. The mean arterial blood pressure (ABP) was recorded in conscious rats using the tail-cuff method (Harvard 50–9331 Rectilinear Recording System; Harvard Apparatus, Kent, UK). Rats were acclimated for restraint and tail-cuff inflation for ≥5 to 7 days before the procedure. A tail-cuff occluder with an optical pulse sensor was placed proximally on the tail to...
measure systolic and diastolic pressure and mean ABP. At least three consecutive readings were obtained and averaged for each rat.

Echocardiography. Echocardiography was performed in all the groups at baseline and at the end (4 wk) of the study. The rats were anesthetized with a mixture of ketamine hydrochloride (25 mg/kg body wt) and xylazine (5 mg/kg body wt). Echocardiograms were recorded with an echocardiography system equipped with a 12-MHz phased-array transducer (Sonos 5500; Hewlett Packard). Two-dimensional short-axis views of the left ventricle and M-mode tracings were recorded through the anterior and posterior LV walls at the level of the papillary muscles to measure LV end-diastolic dimension (LVEDD) and LV end-systolic dimension (LVSD). Fractional shortening (FS) was calculated from these dimensions (32).

Isolated heart perfusion study. Animals were anesthetized using phenobarbital (40 mg/kg body wt ip), and hearts were rapidly excised and immediately placed in ice cold Kreb-Henseleit heparinized solution. The ascending aorta was canulated to attach the heart to a nonrecirculating, constant flow Langendorff apparatus (Radnotti; Harvard Apparatus). Hearts were perfused with a Kreb-Henseleit buffer (pH 7.4) at a constant flow of 13 ml/min at 37°C, and the buffer was aerated with a mixture of 95% O2-5% CO2. A saline-filled latex balloon connected to a pressure transducer (Gold Statman) was inserted into the left ventricle. The balloon was filled with enough saline to produce an end-diastolic pressure of 16–18 mmHg. Digital analysis of the pressure wave was performed and displayed by an electronic polygraph (NEC-San-eti, 2238). LV functions were assessed by recording: LV developed pressure (LVDP; peak systolic minus end diastolic pressure); LV end-diastolic pressure (LVEDP); maximum rate of pressure rise (dp/dtmax); and rate pressure product (RPP = heart rate × LVDP).

After the measurement of baseline values, ischemia was achieved by clamping the aortic cannula to achieve zero flow for 30 min. Hearts were then reperfused with the same Kreb-Henseleit solution, and postischemic contractile parameters were recorded at 120 min of reperfusion. At the end of experimental period, heart samples were collected and immediately stored at −80°C for immunohistochemical analysis.

Immunohistochemistry. Ventricular samples were fixed in phosphate-buffered formalin (pH 7.4) for 24 h and then embedded in paraffin wax. Five-micrometer-thick sections were cut and placed on sialinized slides (Biogenex), air dried overnight at room temperature, incubated at 60°C for 20 min, dewaxed in xylene, and rehydrated using graded alcohol concentrations. For antigen retrieval, samples were boiled for 10 min in antigen retrieval solution (0.1 m citric acid + 0.1 m sodium citrate buffer solution, pH 6). Slides were washed with dH2O, incubated with blocking serum for 30 min (Ultra Blocks; Biogenex), washed with dH2O, incubated with Hoescht 33258 (1 μg/ml) for 10 min in a humidified chamber, protected from light, at 37°C. After being stained, the culture plates were examined using fluorescent microscope (Olympus BX 51). A total of five different fields per dish was counted to quantify the number of cells containing fragmented nuclei.

Apoptosis in cardiomyocytes. Cardiomyocyte apoptosis was studied after the cells were stained with Hoescht 33258. For this, the cardiomyocytes from different groups in culture dishes were washed three times with PBS and incubated with 10 μM solution of the fluorescent probe 5-(6)-chloromethyl-2′,7′-dihydrodcoleorescin diacetate (Molecular Probes, Eugene, OR) at 37°C for 30 min in a humidified chamber. Fluorescent images of 100 cells from multiple fields per dish were recorded with the Olympus BX 51 fluorescent microscope. Fluorescence intensity was measured using digital image processing software (Image Pro Plus).

Measurement of oxidative stress in cardiomyocytes. The level of oxidative stress in isolated cardiomyocytes was monitored by the measurement of production of reactive oxygen species (ROS). Cardiomyocytes from different treatment groups in the culture dishes were washed with PBS and incubated with 10 μM solution of the fluorescent probe 5-(6)-chloromethyl-2′,7′-dihydrodcoleorescin diacetate (Molecular Probes, Eugene, OR) at 37°C for 30 min in a humidified chamber. Fluorescent images of 100 cells from multiple fields per dish were recorded with the Olympus BX 51 fluorescent microscope. Fluorescence intensity was measured using digital image processing software (Image Pro Plus).

In Vitro Studies

Isolation of adult ventricular cardiomyocytes. Cardiomyocytes were isolated from normal adult male rats using Langendorff perfusion apparatus as described previously (11). Cardiomyocytes (1 × 106 per well) were plated on laminin-coated polystyrene tissue culture dishes. Plated cells were incubated in serum-free culture medium M199 supplemented with antibiotics (streptomycin/penicillin, 100 μg/ml) at 37°C under a 5% CO2-95% air atmosphere. Two hours after plating, the culture medium was changed to remove unattached dead cells and the viable cardiomyocytes were incubated overnight under the same culture conditions.

Cardiomyocyte treatment. After an initial incubation of 24 h, >90% of cardiomyocytes were viable and these cells in culture dishes were allocated into the following groups: control group (n = 5) received saline; EPO group (n = 5) received EPO (5 U/ml) for 48 h with the fresh addition of EPO after 24 h; AD group (n = 5) cardiomyocytes received doxorubicin (5 μM) for 24 h; and EPO-AD group (n = 5) cardiomyocytes received both EPO (5 U/ml) and doxorubicin (5 μM) after 24-h doxorubicin treatment was stopped and fresh medium containing EPO (5 U/ml) was added to continue with EPO treatment for next 24 h.

This dose and treatment protocol is chosen following the completion of a separate pilot study (data not shown) based on previously published data (7, 18).

In general data collection, we observed a significant gain (P < 0.05) in the body weight in control as well as in EPO-treated rats. However, in AD-treated animals we did not observe any significant change in the weight after 4 wk. Treatment with EPO in AD group resulted in a normal weight gain (Fig. 1A).
wk, the HCT level significantly decreased \((P < 0.05)\) in the AD-treated group compared with control animals. However, EPO treatment significantly prevented the AD-induced decrease in HCT level (Fig. 1C).

\section*{Cardiac Function}

AD exposure significantly decreased \((P < 0.05)\) %FS values after 4 wk of treatment, whereas treatment with EPO prevented the AD-induced decrease in %FS. AD treatment did not induce an increase in LVEDD levels. Neither EPO treatment alone nor EPO and AD treatment altered LVEDD values. LVSD levels increased significantly \((P < 0.05)\) in the AD-treated group, and EPO-AD treatment prevented the increase in LVSD levels (Fig. 2, A–C).

\section*{Isolated Heart Perfusion}

Isolated hearts from each treatment group were perfused, and baseline levels of heart rate, LV systolic pressure (LVSP), LVEDP, LVDP, and \(dp/dt\) were recorded. No difference in heart rate values was observed in AD- and EPO-treated groups compared with control. AD exposure significantly decreased LVSP and LVDP levels and increased the LVEDP values. EPO-AD treatment normalized the AD-induced changes in these parameters. Myocardial function was also evaluated by measuring \(dp/dt\) levels, which were significantly decreased with AD treatment, and EPO-AD treatment recovered the AD-induced decrease in \(dp/dt\) levels. RPP levels in AD-treated animals were significantly decreased compared with the control group, whereas EPO-AD treatment prevented AD-induced decrease in RPP levels (Fig. 3, A–F).

Isolated hearts (after baseline measurements) were also subjected to global ischemia by clamping the aortic cannula to achieve zero flow for 30 min, and then contractile parameters were assessed after 120 min of reperfusion. After ischemia-reperfusion, no changes were found in heart rate in AD- or...
EPO-treated groups compared with the control group. LVSP and LVDP significantly decreased after ischemia-reperfusion in AD-treated group. EPO administration prevented the decrease in the LVSP and LVDP levels in the EPO-AD compared with the AD-treated animals (Fig. 3). Similarly EPO-AD treatment significantly improved the AD-induced postischemic changes in LVEDP, dp/dt, and RPP levels (Fig. 3, A–F).

**Oxidative Stress and Apoptosis**

To study the mechanism of EPO-mediated protection in doxorubicin-induced cardiac dysfunction, we assessed the level of oxidative stress and apoptosis in cardiomyocytes after treatment with AD and EPO. The level of oxidative stress in terms of production of intracellular ROS was significantly higher in AD-exposed cardiomyocytes (Fig. 4). However, the AD-induced increase in ROS production was significantly prevented by EPO treatment (Fig. 4).

Doxorubicin exposure significantly increased the level of cardiomyocytes apoptosis, which was associated with increase in the ratio of Bax to Bcl2. Treatment with EPO prevented doxorubicin-induced cardiomyocyte apoptosis and an increase in Bax-to-Bcl2 ratio (Fig. 5). We also observed caspase 3 activation in doxorubicin-treated cardiomyocytes as we detected a 17-kDa cleaved caspase-3 fragment in AD-exposed cardiomyocytes. Treatment with EPO prevented the AD-induced activation of caspase-3 (Fig. 5).

**Angiogenesis**

The role of EPO in maintaining the coronary microvasculature in doxorubicin-induced heart failure was assessed by immunohistochemical detection of the angiogenic marker CD31 in the myocardium. The number of CD31-positive capillaries was counted in five fields in one section. AD treatment decreased the number of CD31-positive capillaries (2.4 ± 0.34 vs. 4.1 ± 0.34 capillaries/high-power field in control; $P < 0.05$). Cotreatment with EPO significantly prevented the AD-induced decrease in the number of CD31-positive capillaries (5.4 ± 0.33 vs. 2.4 ± 0.34 capillaries/high-power field; $P < 0.05$; Fig. 6).

**DISCUSSION**

The present study examined the possible protective effect of EPO on cardiac function in a rat model of doxorubicin (AD)-induced heart failure. Our data indicate that EPO administra-
tion for 4 wk improved cardiac function during doxorubicin-induced heart failure, as measured by echocardiography as well as improvement in contractile parameters in isolated perfused hearts. The preservation of heart function was associated with a decrease in the level of oxidative stress and apoptosis in cardiomyocytes as well as a significant increase in number of capillaries in the EPO-AD-treated group. So far, the antiapoptotic effects of EPO in doxorubicin-induced cardiomyopathy have been demonstrated in neonatal cardiomyocytes. Our study in adult rats demonstrates for the first time that EPO administration has a protective role in the setting of doxorubicin-induced heart failure by preventing the doxorubicin-induced increase in oxidative stress and apoptosis in cardiomyocytes.

Doxorubicin is well known for its cardiac toxicity during chemotherapy for cancer patients. Doxorubicin-induced heart failure has been characterized by thinning and dilatation of the ventricular wall and a reduced ejection fraction. In animal models, a similar response has been reported (10, 26, 36, 37). In the present study, AD exposure resulted in a significant decrease in HCT levels (35), reducing the oxygen-carrying capacity of the blood. EPO treatment in the AD group restored the HCT levels to normal and was not associated with an unnecessary elevation in HCT levels. The echocardiographic evaluation demonstrated a significant reduction in FS in the AD group, which was similar to the response reported by other investigators after 2 and 4 wk of treatment (36). When EPO was coadministered with doxorubicin and continued for 4 wk, there was a significant improvement in arterial blood pressure and FS. These results suggest that EPO prevented the AD-induced deterioration in heart function. Multiple mechanisms have been proposed to explain the protective effects of EPO in doxorubicin-induced cardiotoxicity. Doxorubicin depletes GATA-4, a key regulator of heart development, and EPO has been shown to restore GATA-4 expression in doxorubicin-induced cardiomyopathy (1, 19, 25). Furthermore, EPO restores the doxorubicin-induced alterations in cardiomyocyte sarcomeric proteins, including myosin heavy chain, troponin I, and desmin. These proteins are important for the structural integrity and contractile function of cardiomyocytes (21, 23).

To exclude the possibility of involvement of systemic factors in EPO modulation of AD-induced alterations in cardiac performance, we evaluated the levels of various contractile parameters in isolated perfused hearts from different treatment groups. The AD-treated group had a significant decline in baseline levels of LVDP, dP/dt max, and RPP compared with the control group and EPO cotreatment improved these AD-induced changes. Previous studies (21) in mice also demonstrated that doxorubicin induced deterioration of cardiac function in terms of LV fractional shortening, dP/dt, and increase in LV diameter and LVEDP was prevented by EPO treatment. Functional improvement seen in the baseline measurements was also observed after 30 min of ischemia and 120 min of reperfusion. This effect of EPO is consistent with the previous studies that demonstrated the protective effect of EPO after regional ischemia and myocardial infarction in experimental models and in clinical reports. Cai et al. (5) demonstrated that a single dose of EPO 24 h before global ischemia in normal hearts significantly improved posts ischemic function of the isolated rat heart. We (3) recently reported that exposure to acute intermittent hypoxia increased endogenous cardiac EPO expression, which was associated with a better recovery of posts ischemic function.

To delineate the mechanisms for this EPO-mediated protection in the doxorubicin-induced deterioration of cardiac function in the present study, we assessed the level of oxidative stress and apoptosis in isolated cardiomyocytes. We observed a significant increase in the level of ROS in doxorubicin-treated cardiomyocytes. EPO treatment in our studies prevented doxorubicin-induced increase in ROS levels; in this regard Kim et al. (18) demonstrated that EPO treatment has protective role in doxorubicin-induced heart failure by decreasing the level of oxidative stress. Oxidative stress is known to
induce apoptosis in a variety of cell types by activating several proapoptotic pathways and by downregulating several antiapoptotic parameters. We (11, 12) recently reported in adult rat cardiomyocytes that TNF-α/H9251-induced oxidative stress in cardiomyocytes leads to apoptosis by increasing the phosphorylation of p38 MAP kinase and activation of NF-κB pathway. In the present study, apoptosis was more pronounced in doxorubicin-treated group and EPO treatment decreased the level of cardiomyocyte apoptosis. Several reports suggest that apoptosis contributes to the cardiac dysfunction in doxorubicin-induced cardiomyopathy. Delivery of EPO at the time of coronary ligation to normal hearts improved postischemic cardiac functions, reduced infarct size, and was associated with less tunnel-positive cells in LV samples (31). Basically, EPO inhibits caspase-3 activity by blocking cytochrome c release in the mitochondria (37). In this regard, it has also been demonstrated in isolated neonatal cardiomyocytes that EPO protects against doxorubicin-induced apoptosis by activating phosphatidylinositol 3-kinase-AKT cell survival pathway (6, 18). However, to the best of our knowledge, the present study is the first to demonstrate protective effect of EPO in doxorubicin-induced heart failure by preventing an increase in oxidative stress and apoptosis in adult cardiomyocytes.

Doxorubicin decreased the number of capillaries in the heart as determined by the decreased level of the angiogenic marker CD31. EPO treatment improved neovascularization in doxorubicin-induced heart failure in rats, as evidenced by preservation of the number of CD31-positive capillaries in the EPO-AD-treated group. Hamed et al. (17) showed that EPO treatment in rats for 7 wk was potentially protective against doxorubicin-induced myocardial dysfunction. These effects were mediated by enhancement in the number of endothelial progenitor cells, their capacity for tube formation, and an increase in capillary density. EPO stimulated neo-vascularization by inducing direct mitogenic effects on endothelial cells through local upregulation of VEGF (37). In a mice model of burn wounds, treatment with recombinant human EPO (rHuEPO) increased the expression of CD31, improved angiogenesis and wound healing by increasing the production of VEGF and nitric oxide in the wound area (15). In the present study, we also observed a significant increase in CD31 expression in our “EPO control” group. In this regard, it has been observed that rHuEPO
treatment in a cyclosporine-induced nephrotoxicity model in rat increased CD31 expression in control group; this finding has been explained by the fact that tubular cells in the kidney as well as other nonhematopoietic cells also express EPO receptor (13).

One potential disadvantage of EPO treatment in patients is an unwanted elevation of HCT after long-term treatment with EPO. This could lead to hypertension, seizures, and vascular thrombosis. In the present study in our “only EPO”-treated animals, we observed a significant increase in HCT levels. In this regard, Hamid et al. (17) found that EPO treatment improved the myocardial performance in doxorubicin-induced cardiomyopathy, which was associated with an increase in HCT levels in their EPO control rats (23). In another study, it has been found that low-dose of EPO improves cardiac function in experimental heart failure without increasing HCT levels; however, some of the beneficial effects were less pronounced in the “low-dose group” than “high dose group” in this study, which might indicate that some of the beneficial effects are related to increased HCT levels and consequently increased oxygen delivery (22). Thus the optimal dose and timing of EPO treatment during chemotherapy are very important and still need to be defined. EPO in combination with other cardioprotectants may synergistically activate cardioprotective pathways that allow a lower dose of EPO to be used.

Another option to circumvent the unwanted effects of EPO on HCT could be the use of recently discovered nonerythropoietic derivatives of EPO, which retain its tissue-protective properties without the undesired effect on erythropoiesis. In this context, carbamylated EPO (20) and asialo-EPO (14) have been identified, which are nonerythropoietic but retain the protective action of EPO in different animal models, including cerebral ischemia, spinal cord injury, and diabetic neuropathy. Basically, these derivatives of EPO do not bind to classical EPO receptor but still retain the cytoprotective properties. Both EPO and carbamylated EPO have very similar pharmacoki-
netic variables and plasma half-life in particular (20) in contrast to asialo-EPO that has a very short half-life (14). In a recent study, it has been observed that both EPO and carbamylated EPO have neuroprotective effects in cisplatin-induced neurotoxicity. It is important to note that both the molecules had similar protective effects (4). These findings open up the possibility of distinguishing between the tissue-protective action of EPO and its potentially detrimental effects. Moreover, these preclinical findings suggest that these nonerythropoietic tissue-protective derivatives of EPO, which appear to elicit fewer adverse effects, may be especially useful in clinical settings.

ACKNOWLEDGMENTS

We thank Dr. Ren-Ke Li from Toronto General Hospital for critical reading and helpful discussions of the manuscript.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


