Potent Metalloporphyrin Peroxynitrite Decomposition Catalyst Protects Against the Development of Doxorubicin-Induced Cardiac Dysfunction

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Background—Increased oxidative stress and dysregulation of nitric oxide have been implicated in the cardiotoxicity of doxorubicin (DOX), a commonly used antitumor agent. Peroxynitrite is a reactive oxidant produced from nitric oxide and superoxide in various forms of cardiac injury. Using a novel metalloporphyrinic peroxynitrite decomposition catalyst, FP15, and nitric oxide synthase inhibitors or knockout mice, we now delineate the pathogenetic role of peroxynitrite in rodent models of DOX-induced cardiac dysfunction.

Methods and Results—Mice received a single injection of DOX (25 mg/kg IP). Five days after DOX administration, left ventricular performance was significantly depressed, and high mortality was noted. Treatment with FP15 and an inducible nitric oxide synthase inhibitor, aminoguanidine, reduced DOX-induced mortality and improved cardiac function. Genetic deletion of the inducible nitric oxide synthase gene was also accompanied by better preservation of cardiac performance. In contrast, inhibition of the endothelial isoform of nitric oxide synthase with N-nitro-L-arginine methyl ester increased DOX-induced mortality. FP15 reduced the DOX-induced increase in serum LDH and creatine kinase activities. Furthermore, FP15 prevented the DOX-induced increase in lipid peroxidation, nitrotyrosine formation, and metalloproteinase activation in the heart but not NAD(P)H-driven superoxide generation. Peroxynitrite neutralization did not interfere with the antitumor effect of DOX. FP15 also decreased ischemic injury in rats and improved cardiac function and survival of mice in a chronic model of DOX-induced cardiotoxicity.

Conclusions—Thus, peroxynitrite plays a key role in the pathogenesis of DOX-induced cardiac failure. Targeting peroxynitrite formation may represent a new cardioprotective strategy after DOX exposure or in other conditions associated with peroxynitrite formation, including myocardial ischemia/reperfusion injury. (Circulation. 2003;107:896-904.)

Key Words: cardiac function; doxorubicin; oxidative stress; nitric oxide; heart failure

Doxorubicin (DOX; Adriamycin) is a broad-spectrum antitumor anthracycline antibiotic that is commonly used to treat a variety of cancers, including severe leukemias, lymphomas, and solid tumors.1–4 The clinical use of DOX is limited because of its severe cardiotoxic side effects: irreversible degenerative cardiomyopathy and chronic heart failure.3,4 The cardiotoxicity of DOX involves increased oxidative stress in cardiomyocytes, alteration of cardiac energetics, and a direct effect on the DNA.5–11 The production of peroxynitrite, a reactive oxidant formed from the rapid reaction of nitric oxide (NO) and superoxide, was recently demonstrated in rodent models of heart failure.10–13 Using a novel metalloporphyrinic peroxynitrite decomposition catalyst molecule, we have now directly tested the potential pathogenetic role of peroxynitrite in a DOX-induced acute and chronic cardiac dysfunction and heart failure in acute and chronic murine models.

Methods

The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985) and was per-
formed with the approval of the local Institutional Animal Care and Use Committee.

**Animals**

Male BALB/c, C57BL/6 (inducible nitric oxide synthase [iNOS]−/−), or C57BL6-NOSII (iNOS−/−) mice (Jackson Laboratories, Bar Harbor, Me) weighing 25 to 35 g were given a single dose of DOX HCl (Sigma/Aldrich) at 25 mg/kg IP and used for biochemical measurements at 2 days and for functional measurements at 5 days as described previously.10,14 In a separate set of experiments, DOX was injected in 3 equal doses of 9 mg · kg−1 · d−1 on days 1, 10, and 20, and hemodynamics was measured on day 25. Treatment with FeCl tetrakis-2-(triethylene glycol monomethyl ether) pyridyl porphyrin (FP15) (0.03 to 1 mg · kg−1 · d−1 PO), aminoguanidine (AG; 50 and 100 mg · kg−1 · d−1 IP), or N-nitro-L-arginine methyl ester (L-NAME, 10 and 20 mg · kg−1 · d−1 IP) started 2, 24, and 24 hours before DOX injection and continued until hemodynamic measurements were completed or survival studies were terminated.

**Hemodynamic Measurements in Mice**

Five days after DOX administration in the acute model or on day 25 in the chronic model, left ventricular (LV) performance was analyzed in mice anesthetized with injections of ketamine (80 mg/kg IP) and xylazine (10 mg/kg IP). A micropump pressure-volume catheter (SPR-839; Millar Instruments) was inserted into the right carotid artery and advanced into the LV under pressure control as described.14,15 After stabilization for 20 minutes, the signals were recorded continuously with an ARIA pressure-volume conductance system (Millar Instruments) coupled with a Powerlab/4SP A/D converter (AD Instruments), stored, and displayed on a personal computer. The heart rate, maximal LV systolic (LVSP) and end-diastolic (LVEDP) pressures, maximal slope of systolic pressure increment (+dP/dt) and diastolic pressure decrement (−dP/dt), stroke volume (SV), stroke work (SW), ejection fraction, and cardiac output (CO) were calculated and corrected according to in vitro and in vivo volume calibrations with a cardiac pressure-volume analysis program (PVA-N29; Millar Instruments). After these measurements, the catheter was pulled back into the aorta for the measurement of mean arterial blood pressure (mean BP).

**Malondialdehyde Assay**

Malondialdehyde (MDA) formation was used to quantify the lipid peroxidation in tissues and was measured as thiobarbituric acid-reactive material from heart homogenates as described.16

**Xanthine Oxidase Assay and NAD(P)H Oxidase Assay**

NAD(P)H oxidase and xanthine oxidase activities in heart homogenates were measured by the lucigenin chemiluminescence method of Mohazzab et al.17 modified to the use of 10 μmol/L lucigenin. Protein content was measured in an aliquot of the homogenate by the Lowry method.

**Immunohistochemistry**

Paraffin-embedded 3-μm sections were processed for immunohistochemical determination of 3-nitrotyrosine (3-NT) as described.13,18

**Serum LDH and Creatine Kinase Measurement**

Forty-eight hours after DOX treatment, serum LDH and creatine kinase (CK) activities were determined by end-point activity assay kits (Sigma Diagnostics).14

**Metalloproteinase Zymography**

Forty-eight hours after DOX treatment, hearts were homogenized and used for matrix metalloproteinase (MMP) zymography as described.14

**Survival Studies**

Animals exposed to an acute dose of DOX (aDOX; 25 mg/kg IP, n = 180) received either FP15 (0.3, 0.1, 0.03 mg · kg−1 · d−1 PO), AG (50 and 100 mg · kg−1 · d−1 IP), L-NAME (10 and 20 mg · kg−1 · d−1 IP), or vehicle (isotonic saline, 0.2 mL PO or IP) starting 2, 24, and 24 hours before DOX injection, respectively. Mortality was monitored and recorded twice daily for 7 days. In a separate set of chronic experiments, DOX (cDOX, n = 120) was injected in 3 equal doses of 9 mg · kg−1 · d−1 every 10 days, and survival was followed for 30 days in the presence of FP15 (1, 0.1, 0.01, 0.03 mg · kg−1 · d−1 PO) or vehicle treatment.

**Mouse Breast Carcinoma Model**

The effect of FP15 on tumor growth and the antitumor effect of DOX in a mouse model of breast cancer were investigated in 4T1 mammary adenocarcinoma cells.19 Cells (n = 10) were injected into the mammary fat pad of female BALB/c mice. Fifteen days later, mice were randomized into 4 groups (n = 10 per group) and received FP15 (1 mg · kg−1 · d−1 PO), DOX (4 mg · kg−1 · d−1 IP twice a week), DOX + FP15, or vehicle. Tumor diameters (x, y, and z) were recorded twice a week, and tumor size was estimated in mm3.

**Myocardial Ischemia/Reperfusion**

Twenty male Wistar rats (Charles River) weighing 300 to 330 g were anesthetized with thiopentone sodium (60 mg/kg IP), tracheotomized, and mechanically ventilated. Myocardial infarction was induced by a 1-hour ligation of the left anterior descending coronary artery, and infarct size and area at risk were quantified by use of the pthalho blue/triphenyl tetrazolium chloride technique as described previously.20 Ten minutes before reperfusion, the rats received an intravenous injection of either FP15 (0.3 mg/kg, n = 8) or vehicle (isotonic saline, 0.5 mL; n = 12).

**Statistical Analysis**

Results are reported as mean ± SEM. Statistical significance between 2 measurements was determined by the 2-tailed unpaired Student’s t test, and among groups it was determined by ANOVA with Bonferroni’s correction. Survival curves were compared by the log-rank test. Probability values of P < 0.05 were considered significant.

**Reagents**

Reagents were obtained from Sigma/Aldrich unless indicated otherwise. The peroxynitrite decomposition catalyst FP15 was synthesized as described.21

**Results**

**Cardiac Function**

Both acute and chronic administration of DOX induced a significant decrease in heart rate, mean BP, LVSP, +dP/dt, −dP/dt, SV, SW, ejection fraction, and CO and an increase in LVEDP, in mice (Figures 1 and 2). Treatment with FP15 (1 mg · kg−1 · d−1 PO) or AG (100 mg · kg−1 · d−1 IP) significantly attenuated the DOX-induced changes in ventricular function. FP15 or AG alone exerted no significant effects on hemodynamic parameters (Figures 1 and 2). Better cardiac function was also seen or AG alone exerted no significant effects on hemodynamic parameters (Figures 1 and 2).

**MDA Formation**

FP15 significantly attenuated the DOX-induced increase in MDA formation in hearts (Figure 3A), indicative of an overall reduction in oxidative stress in the presence of the peroxynitrite decomposition catalyst compound.
Figure 1. Effects of FP15 on DOX-induced acute and chronic cardiac dysfunction. Effect of DOX and FP15 on LVSP, LVEDP, LV +dP/dt, LV –dP/dt, mean BP, heart rate, SV, SW, ejection fraction, and CaO in BALB/c mice. CO, control; aDOX, DOX-treated (single dose of 25 mg/kg IP); CO +FP15, control treated with FP15 (1 mg · kg⁻¹ · d⁻¹ PO for 5 days); aDOX +FP15, treated with DOX (single dose of 25 mg/kg IP) and FP15 (1 mg · kg⁻¹ · d⁻¹ PO for 5 days); cDOX, DOX-treated (3 doses of 9 mg/kg IP every 10th day for 25 days), cDOX +FP15, treated with DOX (3 doses of 9 mg/kg IP every 10th day for 25 days) and FP15 (1 mg · kg⁻¹ · d⁻¹ PO for 25 days). Hemodynamic parameters were measured 5 (aDOX) or 25 (cDOX) days after DOX administration. Results are mean ± SEM of 10 to 14 experiments in each group. *P<0.05 vs CO; #P<0.05 vs aDOX or cDOX.
Figure 2. Effects of pharmacological inhibition or genetic deletion of iNOS gene on DOX-induced acute cardiac dysfunction. Effect of DOX, AG, and genetic deletion of iNOS on LVSP, LVEDP, LV \( \Delta P/dt \), LV \( \Delta P/dt \), mean BP, heart rate, SV, SW, ejection fraction, and CaO in mice. iNOS\(^{+/+}\), control; iNOS\(^{-/-}\), control; iNOS\(^{+/+}\) + AG, control treated with AG (100 mg \( \cdot \) kg\(^{-1} \) \( \cdot \) d\(^{-1} \) IP); iNOS\(^{+/+}\) + DOX, iNOS\(^{+/+}\) treated with DOX (single dose of 25 mg/kg IP); iNOS\(^{+/+}\) + DOX + AG, iNOS\(^{+/+}\) treated with DOX (single dose of 25 mg/kg IP) and AG (100 mg \( \cdot \) kg\(^{-1} \) \( \cdot \) d\(^{-1} \) IP); iNOS\(^{-/-}\) + DOX, iNOS\(^{-/-}\) treated with DOX (single dose of 25 mg/kg IP). Hemodynamic parameters were measured 5 days after DOX administration. Results are mean \( \pm \) SEM of 8 to 11 experiments in each group. *\( P<0.05 \) vs iNOS\(^{+/+}\) or iNOS\(^{+/+}\); #\( P<0.05 \) vs iNOS\(^{+/+}\)/DOX.
Xanthine Oxidase Assay and NAD(P)H Oxidase Assay
In heart samples from DOX-treated mice, NADH- and NADPH-driven increases in lucigenin chemiluminescence were significantly greater than in samples from control mice (Figure 3B). FP15 treatment did not significantly decrease NADH- and NADPH-driven signal, consistently with the concept that it primarily intercepts the reactions of peroxynitrite, which is downstream from the formation of superoxide (Figure 3B). Xanthine oxidase seemed to be a minor source of superoxide in each group (Figure 3B).

Nitrotyrosine Formation
Five days after DOX injection, there was a significant increase in cardiac nitrotyrosine formation (a marker of peroxynitrite formation or, more generally, of nitrosative stress). As expected, nitrotyrosine immunoreactivity was attenuated by FP15 (Figure 4).

Serum LDH and CK
Serum LDH and CK activities were significantly elevated 48 hours after DOX injection compared with the activities measured in the control mice (Figure 5, A and B). FP15 significantly attenuated the DOX-induced elevations in serum LDH and CK activities, indicative of reduced myocardial necrosis (Figure 5, A and B).

Metalloproteinase Zymography
On the gelatin zymography gels, only 1 band was detected with a molecular weight of 34 kDa. Densitometric analysis showed an increase of 327% (P<0.01, n=3) in MMP activity in hearts from DOX-treated mice compared with controls. FP15 treatment of animals resulted in a significant reduction in MMP activity (to 165% of control) (Figure 5C).

Survival Studies
Figure 6 shows the results of acute (A–C) and chronic (D) survival experiments. At 0.1 and 0.3 or 1 mg/kg FP15, a significant protection was noted against DOX-induced mortality in both acute (Figure 6A) and chronic (Figure 6D) models.

To determine the sources of NO that contribute to peroxynitrite formation and associated cytotoxicity, we used a combined approach (pharmacological inhibition and genetically deficient mice). Significant protection against mortality was seen with the iNOS inhibitor AG (100 mg·kg⁻¹·d⁻¹ IP; Figure 6B). In contrast, the primarily constitutive NOS inhibitor L-NAME (10 and 20 mg·kg⁻¹·d⁻¹ IP; Figure 6B) significantly increased mortality. There was no difference in the survival of iNOS⁻/⁻ and iNOS⁺/⁺ mice treated with DOX; L-NAME (20 mg·kg⁻¹·d⁻¹ IP) further aggravated DOX-induced mortality in iNOS⁺/⁺ mice (Figure 6C).
Effect of FP15 on Tumor Growth and Antineoplastic Effect of DOX

FP15, at the highest dose used (1 mg · kg⁻¹ · d⁻¹ PO), was tested on tumor growth and on the antineoplastic activity of DOX, and it failed to affect these parameters, indicating that peroxynitrite formation does not represent an important mechanism for DOX-induced antitumor effects in the present experimental models (Figure 7, A and B).

Effects of FP15 on Myocardial Damage Produced by Transient Coronary Artery Ligation

To test whether the cardioprotective effect of FP15 extends to other forms of myocardial injury associated with peroxynitrite generation, a rat model of acute myocardial infarction was used. The area at risk was comparable in vehicle and FP15 groups (45.9±1.7% versus 46.5±4.3%, respectively). Infarct size was significantly reduced by FP15 (vehicle, 54.2±2.9%; FP15, 40.9±4.3% of area at risk; P=0.018)

Discussion

DOX continues to be an effective and widely used broad-spectrum chemotherapeutic agent. However, its clinical use is limited because of its serious dose-dependent cardiotoxicity. Clinical and experimental investigations suggested that increased oxidative stress associated with an impaired antioxidant defense status plays a critical role in subcellular remodeling, calcium-handling abnormalities, alteration of cardiac energetics, and subsequent cardiomyopathy and heart failure associated with DOX treatment.5,6,8,10–12 Increased iNOS expression and nitrotyrosine formation have been shown in cardiomyocytes of mice 5 days after a single dose of DOX.10,11

Our results indicate that peroxynitrite is formed in the heart after DOX exposure and plays a pathogenetic role in the development of acute and chronic DOX-induced heart failure. Treatment with the peroxynitrite decomposition catalyst FP15 attenuated the development of cardiac dysfunction, increased survival, and reduced the DOX-induced increase in serum LDH and CK activities, consistent with protection against peroxynitrite-mediated myocyte necrosis. FP15 also abolished tyrosine nitration in the hearts of DOX-treated animals. Nitrotyrosine was initially considered a specific marker of peroxynitrite generation. Now it is clear that other pathways can sometimes also induce tyrosine nitration.23 Thus, nitrotyrosine is now generally considered a collective index of reactive nitrogen species.23,24 Nevertheless, the increase in nitrotyrosine in myocytes of DOX-treated mice and its abolishment by FP15 suggest that a causative link exists between oxidative and nitrosative stress and cardiotoxicity of DOX. FP15 also prevented DOX-induced cardiac lipid peroxidation and MMP activation. MMP contributes importantly to the development of various pathophysiological conditions, including dilated cardiomyopathy, congestive heart failure, and reperfusion injury.25–28 Oxidative stress causes tissue injury through activation of the precursors of MMPs (proMMPs). The activation of proMMPs is triggered by peroxynitrite generation via an extensive S-glutathiolation reaction.29 By inhibiting this reaction, peroxynitrite decomposition catalysts may reduce MMP activation. In addition to direct oxidation, peroxidation, and nitration reactions and MMP activation, likely additional downstream cytotoxic mechanisms elicited by peroxynitrite during DOX-induced cardiac injury include DNA injury and activation of the nuclear enzyme poly(ADP-ribose) polymerase, as well as the inhibition of myofibrillar CK.10–14,30

Peroxynitrite is formed from the reaction of superoxide anion and NO. Our results indicate that NAD(P)H oxidase–dependent superoxide generation but not xanthine oxidase upregulation contributes to the DOX-induced increased oxidative stress in the myocardium. The cardiac mitochondria may represent additional sources of superoxide and other oxygen free radicals.3,4,6 With regard to the source of NO, low levels of constitutively produced NO are present in the heart under all conditions. Upregulation of iNOS may represent an additional source of NO during DOX cardiotoxicity.10,11 An inhibitor of iNOS, AG, as well as genetic deletion of the
iNOS gene, preserved cardiac performance in DOX-treated animals and in the case of AG also improved survival in this very severe model. In agreement with our results, Mostafa et al.31 demonstrated in a chronic rat model that AG given concurrently with DOX normalized LDH and lipid peroxidation. Furthermore, AG reduced the mortality and improved the histopathology of the DOX-treated heart. In sharp contrast, an inhibitor of constitutive NOS, L-NAME, aggravated DOX-induced mortality in both BALB/c and iNOS−/− mice. On the basis of these findings, we hypothesize that much of the NO that contributes to peroxynitrite formation is derived from iNOS. The detrimental effects of L-NAME are probably related to the fact that endothelial NOS–derived NO is a maintainer of basal myocardial blood flow and its inhibition leads to severe cardiac ischemia.32 A multitude of compounds that modulate endogenous antioxidant systems or exert anti-

Figure 6. Effects of FP15, AG, L-NAME, or genetic deletion of iNOS on survival in a DOX-induced acute (A–C) or chronic (D) heart failure models in mice. A, Effect of various doses of FP15 (0.03, 0.1, and 0.3 mg · kg⁻¹ · d⁻¹ PO) on DOX-induced mortality (25 mg/kg IP) in mice. B, Effect of various doses of AG (50 or 100 mg · kg⁻¹ · d⁻¹ IP) or L-NAME (10 or 20 mg · kg⁻¹ · d⁻¹ IP) on DOX-induced mortality (25 mg/kg IP) in mice. C, Effect of genetic deletion of iNOS (L-NAME (20 mg · kg⁻¹ · d⁻¹ IP) on DOX-induced mortality (25 mg/kg IP) in mice. D, Effect of various doses of FP15 (0.03, 0.1, and 1 mg · kg⁻¹ · d⁻¹ PO) on DOX-induced chronic mortality (3 doses of 9 mg · kg⁻¹ · d⁻¹ IP every 10 days) in mice.

Figure 7. Effects of FP15 on mouse breast carcinoma growth and antineoplastic effect of DOX. A, CO, controls; CO+FP15, control treated with FP15 (1 mg · kg⁻¹ · d⁻¹ IP); DOX, DOX-treated (twice 4 mg · kg⁻¹ · wk⁻¹ IP); DOX+FP15, treated with DOX (twice 4 mg · kg⁻¹ · wk⁻¹ IP) and FP15 (1 mg · kg⁻¹ · d⁻¹ PO). Tumor growth was individually followed in all mice, and tumor diameters (x, y, and z) were measured twice a week after initiation of treatments. Tumor size was calculated in cubic millimeters and expressed as percentage of increase over time compared with initial size at start of treatment (day 0). Results are mean±SEM of 10 mice in each group. *P<0.05 vs CO. B, Representative pictures show primary solid breast carcinomas in mice 2 weeks after initial treatment. Scale is in millimeters.
oxidant properties have been proposed for the prevention of DOX-induced cardiotoxicity.\textsuperscript{5,8,9,33,34} Nevertheless, the prevention and treatment of DOX-induced cardiomyopathy remains an unresolved clinical problem. Preclinical experimental and clinical studies have shown that the iron-chelating agent dexrazoxane is protective against anthracycline cardio toxicity in various animal models\textsuperscript{33} and humans.\textsuperscript{4} The thiol compound amifostine is also in clinical use.\textsuperscript{34} However, because thiols react very slowly with peroxynitrite,\textsuperscript{35} it is unlikely that thiol compounds (or traditional antioxidants) could be applied at sufficiently high doses to interfere with the rapid reactivity of peroxynitrite. Although we did not directly compare the efficacy of dexrazoxane or amifostine in the present experimental models, overall, the efficacy of FP15 in our model seems to be comparable to or better than the efficacy of many previously published approaches.\textsuperscript{33,34,36}

The fact that FP15 does not interfere with the antitumor actions of DOX provides an additional indication that potent peroxynitrite decomposition catalysts should be tested in additional preclinical and clinical models of DOX toxicity. FP15 is an N-PEGylated-2-pyridyl iron porphyrin that has shown superior performance as a peroxynitrite decomposition catalyst.\textsuperscript{21} Endogenous reducing agents such as ascorbate and glutathione react too slowly with peroxynitrite to complete with trans-membrane diffusion and reactions with metal centers.\textsuperscript{37,38} Because peroxynitrite reacts very efficiently with synthetic metalloporphyrins,\textsuperscript{39} compounds in this class have been investigated as peroxynitrite decomposition catalysts.\textsuperscript{40} Several water-soluble iron\textsuperscript{41} and manganese\textsuperscript{38} porphyrins have shown very high rates of reaction with peroxynitrite. One such porphyrin, FeTMPS, has been shown to reduce carrageenan-induced paw edema and cause reductions in inflammatory mediator production.\textsuperscript{42-44}

Many pathophysiological conditions of the heart are associated with peroxynitrite formation, including acute myocardial infarction, chronic ischemic heart failure, and diabetic cardiomyopathy.\textsuperscript{13,15,21,30} It seems that peroxynitrite decomposition catalysts improve cardiac function and overall outcome in these models. For instance, FP15 reduced myocardial necrosis in our present rat model of acute myocardial infarction (present study) as well as in a recent porcine study.\textsuperscript{45} Furthermore, FP15 significantly improved cardiac function in a diabetic cardiomyopathy model.\textsuperscript{21} These observations, coupled with the protective effect of FP 15 against DOX-induced cardiotoxicity reported here, support the concept that peroxynitrite is a major mediator of myocardial injury in various pathophysiological conditions, and its effective neutralization can be of significant therapeutic benefit.

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