The Endothelin Receptor Blocker Bosentan Inhibits Doxorubicin-Induced Cardiomyopathy

Sandra Bien, 1 Alexander Riad, 1 Christoph A. Ritter, 1,2 Matthias Gratz, 1 Florian Olshausen, 3 Dirk Westermann, 3 Markus Grube, 3 Thomas Krieg, 3 Sabine Ciecholewski, 3 Stephan B. Felix, 1 Alexander Staudt, 2 Heinz-Peter Schultheiss, 2 Ralf Ewert, 3 Uwe Völker, 4 Carsten Tschöpe, 5 and Heyo K. Kroemer 1

1Department of Pharmacology, 2Institute of Pharmacy, Departments of 3Internal Medicine B and 4Functional Genomics, Ernst Moritz Arndt University, Greifswald, Germany; and 5Charité Berlin, Center for Cardiovascular Research, Berlin, Germany

Abstract

Doxorubicin is a frequently used anticancer drug, but its therapeutic benefit is limited by acute and chronic cardiotoxicity, often leading to heart failure. The mechanisms underlying doxorubicin-induced cardiotoxicity remain unclear. It was previously shown in men that doxorubicin leads to increased endothelin-1 plasma levels. In addition, cardiac-specific overexpression of endothelin-1 in mice resulted in a cardiomyopathy resembling the phenotype following doxorubicin administration. We therefore hypothesized that endothelin-1 is involved in the pathogenesis of doxorubicin cardiotoxicity. In mice (C57Bl/10), we found that doxorubicin (20 mg/kg body weight, i.p.) impaired cardiac function with decreased ejection fraction, diminished cardiac output, and decreased end-systolic pressure points recorded by a microconductance catheter. This impaired function was accompanied by the up-regulation of endothelin-1 expression on mRNA and protein level. In vitro investigations confirmed the regulation of endothelin-1 by doxorubicin and indicated that the doxorubicin-mediated increase of endothelin-1 expression involves epidermal growth factor receptor signaling via the MEK1/2-ERK1/2 cascade, which was further confirmed by immunoblotting studies in the left ventricle of treated animals. Pretreatment of mice with the endothelin receptor antagonist bosentan (100 mg/kg body weight, p.o.) strikingly inhibited doxorubicin-induced cardiotoxicity with preserved indices of contractility. Moreover, bosentan pretreatment resulted in reduced tumor necrosis factor-α content, lipid peroxidation, and Bax expression, as well as increased GATA-4 expression. Thus, endothelin-1 plays a key role in mediating the cardiotoxic effects of doxorubicin and its inhibition may be of therapeutic benefit for patients receiving doxorubicin.

Introduction

Doxorubicin is one of the most frequently used anticancer agents in the treatment of both solid and hematologic malignancies. Its therapeutic use is limited by cardiotoxicity categorized as acute or chronic events. The doxorubicin-induced cardiotoxicity is characterized by left ventricular dysfunction, often leading to congestive heart failure (CHF) with poor prognosis (1). Despite its longstanding application for >20 years, the mechanisms underlying doxorubicin cardiotoxicity remain unclear. One hypothesis was that doxorubicin-induced cardiotoxicity is primarily mediated by the generation of reactive oxygen species (ROS) in cardiomyocytes. The application of antioxidants, however, did not prevent doxorubicin-induced cardiomyopathy pointing to a more complex pathogenesis (2).

Notably, elevated endothelin-1 (ET-1) plasma levels were found in patients treated with doxorubicin (3). In addition, mechanistic studies in mice by Yang and colleagues revealed that cardiac-specific overexpression of ET-1 leads to cardiomyopathy and left ventricle (LV) dysfunction combined with inflammatory characteristics such as increased tumor necrosis factor-α (TNFα) content and interstitial inflammatory infiltrates (4). Moreover, ET-1 has been implicated in the progression of CHF because the expression of ET-1 and its receptors are elevated in the myocardium of rats with CHF and in patients with idiopathic dilated cardiomyopathy (5, 6). Taken together, these data suggest the involvement of ET-1 in the development of doxorubicin-induced cardiotoxicity.

In the present study, we could show that ET-1 mRNA and protein expression was up-regulated by doxorubicin in the LV of C57Bl/10 mice. In parallel, cardiac function was strongly impaired with a decrease in ejection fraction and diminished cardiac output. Pretreatment with the endothelin receptor antagonist bosentan (Tracleer) inhibited the cardiotoxic effects of doxorubicin in mice, resulting in reduced TNFα content, lipid peroxidation, Bax expression, and increased GATA-4 expression. In vitro experiments using both primary cardiomyocytes and cell lines confirmed the induction of ET-1 by doxorubicin and elucidated the molecular pathway involving activation of the epidermal growth factor (EGF) receptor and the MEK1/2-ERK1/2 cascade.

Materials and Methods

Chemicals and reagents. Bosentan was obtained from Actelion Pharmaceuticals. The MEK1/2 inhibitor PD98059 and the EGF receptor tyrosine kinase inhibitor AG1478 were from Cell Signalling Technology. Epirubicin was purchased from Pharmacia GmbH. All other chemicals were obtained from Sigma-Aldrich.

Animals. For animal experiments, C57Bl/10ScSn wild-type mice ages 8 to 10 weeks were obtained from the breeding stocks of the Max-Planck-Institut für Immunologie (Freiburg, Germany). In the first study protocol, mice were assigned to two groups: (a) doxorubicin (Doxo Cell; 20 mg/kg single dose, i.p., a dose shown to be cardiotoxic; ref. 7) and (b) the same volume of saline. After doxorubicin injection (1 and 5 days), mice were hemodynamically characterized. For surgical procedures and hemodynamic measurements, animals were anesthetized (thiopental, 125 μg/g, i.p.), intubated and artificially ventilated. As recently described (8), a 1.4 F microconductance...
pressure catheter (ARIA SPR-719; Millar-Instruments, Inc.) was positioned in the LV for continuous registration of LV pressure-volume loops in a closed-chest model.

In the second study, mice were assigned to four groups: (a) doxorubicin (Doxo Cell; 20 mg/kg, single dose, i.p.), (b) the same volume of saline, (c) bosantan (100 mg/kg body weight, p.o., daily from 3 days before doxorubicin application until day 5) plus doxorubicin (Doxo Cell; 20 mg/kg, single dose, i.p.), or (d) bosantan (100 mg/kg p.o., daily for 8 days). The experiments were done in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. NIH (NIH Publication no. 85-23, revised 1985).

**Tissue preparation.** After hemodynamic characterization, hearts were removed and immediately frozen in liquid nitrogen. For immunohistochemical analyses, tissues were embedded in optimum cutting temperature compound (Tissue Tec) and stored at −80°C.

**Cell culture.** The murine cardiomyocyte–derived HL-1 cells were a kind gift from Prof. W. Claycomb (Department of Biochemistry and Molecular Biology, Louisiana State University Medical Center, New Orleans, LA) and were maintained in Claycomb medium (JRH Biosciences) supplemented with 10% FCS (JRH Biosciences), 2 mmol/L of t-glutamine (Seromed-Biochrom KG), 100 μmol/L of arterenol (Sigma Aldrich), and 100 units/mL of penicillin/streptomycin (Seromed-Biochrom KG). The cervix carcinoma cell line HeLa was obtained from American Type Culture Collection and cultured in RPMI 1640 supplemented with 10% FCS and 2 mmol/L of t-glutamine.

Rat ventricular myocytes were isolated as described previously (9). Immediately after the isolation and separation procedure, cells were plated on laminin-coated 12-well plates using creatine (5 mmol/L), L-carnitine (2 mmol/L), and taurine (5 mmol/L)–supplemented medium 199 as cultured in RPMI 1640 supplemented with 10% FCS and 2 mmol/L of t-glutamine.

**RNA preparation and cDNA synthesis.** Heart samples were homogenized and total RNA was prepared using TRIZOL reagent (Invitrogen). RNA from cell culture experiments were isolated using peqGold RNA Pure (peqLab). Total RNA was reverse-transcribed using the TaqMan Reverse Transcription Kit (Applied Biosystems).

**Quantitative real-time PCR.** Quantitative real-time PCR (qPCR) was done on ABI PRISM 7700 sequence detection system (Applied Biosystems). Primer and probe oligonucleotides for human ppET-1 were previously published (11). Murine ppET-1 primer and probe oligonucleotides (forward primer, 5′-TGTGTTGACATCTCCAAGG-3′; reverse primer, 5′-AGCTCGG-GTGCTGAGTCTGGG-3′; probe, 5′-6FAM-CTCGAGACACGGCTG-3′) were designed based on the reference sequence U35233. Rat ppET-1 primer and probe oligonucleotides (forward primer, 5′-TG CATGATCTGGTGCAACA-3′; reverse primer, 5′-GGT GAGGCAAGGGCTC-3′; probe, 5′-6FAM-TGCGAGGCTGCTCCGTGA-3′) were designed based on the reference sequence NM_015254. For normalization, 18S RNA TaqMan PreDeveloped Assay Reagent (Applied Biosystems) was used and signals were applied to a cloned standard of the respective gene and 18S RNA.

**Preparation of protein extracts.** Cells were harvested in PBS and pelleted by centrifugation at 6,000 × g. Pellets were resuspended in 150 μL of lysis buffer (pH 7.4; 50 mmol/L Tris-HCl, 100 mmol/L NaCl, 0.1% Triton X-100, and 5 mmol/L EDTA) supplemented with protease inhibitors (1 mg/mL aprotinin, 0.5 mg/mL leupeptin, and 100 μmol/L phenylmethylsulfonyl fluoride) and incubated for 45 min on ice. After a centrifugation step at 6,000 × g, supernatant was used for the determination of protein content by the bicinchoninic acid (BCA) method.

For preparation of membrane fractions, the cell pellets were resuspended in 5 mmol/L of Tris-HCl (pH 7.4) supplemented with protease inhibitors, snap-frozen in liquid nitrogen and thawed at 37°C four times. Crude membranes were isolated by a centrifugation step at 100,000 × g for 30 min. The supernatant was collected and pellets were resolved in 5 mmol/L of Tris-HCl supplemented with protease inhibitors. Protein concentration was measured according to the BCA method.

For tissue protein extraction, LV samples were homogenized in 150 μL of Tris-sucrose (pH 7.4) and centrifuged for 1.5 min at 1,500 rpm. Thereafter, lysates were snap-frozen in liquid nitrogen and thawed at 37°C four times, followed by a centrifugation step at 4°C. The supernatant was collected and protein concentration was measured according to the BCA method.

**Immunofluorescence microscopy.** Cryopreserved sections (7 μm) were generated via a CM1900 cryostat (Leica). Protein localization was investigated by confocal laser scanning immunofluorescence microscopy. For ET-1 detection, the Endothelin-1 Immunohistochemistry Staining Kit (Bachem AG) was used according to the manufacturer’s protocol with the exception of the secondary antibody. As secondary antibody, Alexa Fluor 488-labeled IgG (anti-rabbit IgG; Invitrogen) was used. Staining of actin filaments was done using Alexa Fluor 647 Phalloidin (Invitrogen). After staining, slides were mounted in DAKO Fluorescent Mounting Medium (Dako Cytomation).

**Western blot analysis.** Immunoblotting was done using standard techniques. The following antibodies were used: anti-phospho-MEK1/2, anti-MEK1/2, anti-phospho-ERK1/2 (all from Cell Signaling Technology), anti-ERK1/2 (Promega), anti-EGF receptor and anti-phospho-EGF receptor (Upstate Biotech), anti-Bax and anti-Bcl-xL (Santa Cruz Biotechnology), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Biosign International). As secondary antibodies, we used goat anti-mouse or swine anti-rabbit antibody conjugated with alkaline phosphatase (DAKO). Blots were visualized with LumiPhos reagent (Pierce). Before reincubation with another antibody, membranes were stripped in 62.5 mmol/L of Tris-HCl, 2% SDS (pH 6.8) for 15 min at 52°C. Densitometric evaluation was done using the program ImageQuant.

**Determination of ET-1 and TNFα by ELISA.** Myocardial ET-1 and TNFα were measured by ELISA (R&D Systems) according to the manufacturer’s instructions using protein extracts from cells or tissues. Values were normalized to the protein content.

**Lipid peroxidation.** Lipid peroxidation in LV was measured with the commercially available colorimetric assay kit Bioxytech LPO-586 (Oxis International) according to the manufacturer’s instructions. For the calculation of lipid peroxidation, samples were applied to a MDA standard curve and normalized to the relative protein content.

**Statistical analysis.** Statistical analysis of animal experiments was done using nonparametric Kruskal-Wallis and Mann-Whitney U test to compare groups shown as the median as well as the 25th and 75th percentiles. Student’s t test was employed for comparison of the in vitro experiments. *P < 0.05* was considered statistically significant. All statistical analyses were done using SPSS version 12.0 (SPSS Inc.).

**Results**

**ET-1 induction by doxorubicin in vivo.** After doxorubicin administration (1 and 5 days), LV of C57Bl/10 mice were used to determine the prepro-ET-1 (ppET-1) mRNA and ET-1 peptide content. As early as 24 h after doxorubicin administration, a significant increase of ppET-1 mRNA from 0.57 (0.38–1.43) × 10⁻⁷ to 1.59 (1.23–4.28) × 10⁻⁷ was observed. This up-regulation of ppET-1 mRNA was further enhanced 5 days after doxorubicin application to 2.17 (1.77–4.84) × 10⁻⁷ (Fig. 1A). On the protein level, doxorubicin treatment resulted in a significant increase of ET-1 peptide level after 1 day from 0.17 (0.10–0.18) pg/mg (control) to 0.29 (0.21–0.30) pg/mg (doxorubicin). Five days after doxorubicin application, the ET-1 peptide content was significantly increased as well, with a value of 0.31 (0.23–0.35) pg/mg (Fig. 1A).

The detection of ET-1 peptide in cryopreserved slides of LV of C57Bl/10 mice confirmed the increase in ET-1 expression after 5 days of doxorubicin treatment. In control animals, there was only a slight staining of ET-1 recognizable by the green fluorescence, which was markedly increased after doxorubicin treatment (Fig. 1B). For counterstaining of cells, we used phalloidin-staining of actin filaments (red fluorescence).

**ET-1 induction by doxorubicin in vitro.** For investigation of the doxorubicin effect in vitro, we used the murine cardiomyocyte cell line HL-1, the cervix carcinoma cells HeLa and adult rat cardiomyocytes. Figure 1C and D show the results for ET-1 expression analysis in adult rat cardiomyocytes. After 48 h,
0.1 μmol/L of doxorubicin leads to a significant increase from 0.95 (0.44–6.83) × 10⁻⁸ in control cells to 2.58 (1.03–10.51) × 10⁻⁸ in doxorubicin-treated cardiomyocytes. Determination of the ET-1 peptide content confirmed the ET-1 induction by doxorubicin. After an incubation period of 48 and 72 h, an increase in ET-1 peptide from 8.03 (7.75–9.30) and 10.56 (8.41–18.92) pg/mg in control cardiomyocytes to 14.66 (12.62–19.43) and 20.16 (12.82–24.92) pg/mg in doxorubicin-treated cells was observed, respectively. Immunofluorescence staining after removing the respective negative control staining (without primary antibody) confirmed the induction of ET-1 by doxorubicin seen with ELISA experiments (Fig. 1D).

In HL-1 cells, treatment with doxorubicin at concentrations of 0.1, 0.3, and 1 μmol/L for 48 h resulted in a significant regulation of ppET-1 mRNA from 0.16 (±0.08) × 10⁻⁷ (control) to 0.25 (±0.18) × 10⁻⁷, 0.49 (±0.22) × 10⁻⁷, and 0.83 (±0.49) × 10⁻⁷, respectively. Induction of ET-1 expression by doxorubicin in HL-1 cells was further confirmed on the protein level. Untreated control HL-1 cells showed an intracellular ET-1 content of 10.2 (±0.54) pg/mg, which was increased with 0.1 and 1 μmol/L of doxorubicin up to 15.7 (±3.33) and 27.11 (±7.97) pg/mg, respectively (data for HL-1 cells are not shown). In HeLa cells, we also observed a concentration-dependent increase of ppET-1 mRNA expression after 48 h of doxorubicin treatment. Maximum effects on ppET-1 mRNA expression were observed at 1 μmol/L of doxorubicin with a value of 6.17 (±1.11) × 10⁻⁵ versus 0.84 (±0.13) for control cells. On protein level, the maximum effect was also observed at 1 μmol/L of doxorubicin with an increase in intracellular ET-1 from 19.79 (±3.49, control) to 64.03 (±12.13) pg/mg (data for HeLa cells are not shown).

**Involvement of EGF receptor and MEK1/2-ERK1/2 cascade.**

The influence of doxorubicin on phosphorylation of the EGF receptor was studied by immunoblotting. As shown in Fig. 2A, treatment of mice with doxorubicin for 3 h resulted in a strong phosphorylation of the cardiac EGF receptor with an increase in arbitrary units (au, determined by densitometry and normalization
and of 0.33 (±0.07) to 0.88 (±0.15) in HeLa cells, respectively; $P < 0.05$. Elevated phosphorylation of ERK1/2, the kinase directly downstream of MEK1/2, could also be shown [densitometric values of 0.17 (±0.02) to 0.28 (±0.02) in HL-1 cells and of 0.42 (±0.33) to 1.32 (±0.56) in HeLa cells; $P < 0.05$, Fig. 3B]. Pretreatment of HL-1 cells with the MEK1/2 inhibitor PD98059 (10 μM/L) resulted in a decrease of ppET-1 mRNA induction from 0.17 (±0.03, doxorubicin) $\times 10^{-7}$ to 0.13 (±0.03, doxorubicin+PD98059) $\times 10^{-7}$ (Fig. 3C). Accordingly, the doxorubicin-mediated increase of ppET-1 mRNA expression in adult rat cardiomyocytes was significantly decreased by PD98059 from 3.40 (1.16–9.64) $\times 10^{-8}$ (doxorubicin alone) to 1.05 (0.92–8.12) $\times 10^{-8}$ (doxorubicin+PD98059; Fig. 3D). Inhibition experiments in HeLa cells with PD98059 (10 μM/L) resulted in a significantly reduced ppET-1 mRNA induction from 7.65 (±2.86) $\times 10^{-8}$ (doxorubicin) to 4.42 (±1.65) $\times 10^{-6}$ (doxorubicin + PD98059).

**Hemodynamic effects of doxorubicin.** Hemodynamic measurements done 1 and 5 days after doxorubicin administration are summarized in Table 1. One day after doxorubicin application, both systolic and diastolic LV function indexed by dp/dt max (−30%) and dp/dt min (+22%) was impaired. These parameters deteriorated progressively until day 5 after doxorubicin administration compared with nontreated controls (dp/dt max, −43% and dp/dt min +39%). At this time point, myocardial stiffness as an index of diastolic function was enhanced to ~350% in doxorubicin-treated mice compared with controls. Systolic and diastolic LV dysfunction at day 5 after doxorubicin application led to impaired global LV function indexed by heart rate (−21%), stroke volume (−47%), and cardiac output (−60%).

<table>
<thead>
<tr>
<th>Table 1. Influence of doxorubicin on hemodynamic parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 8)</td>
</tr>
<tr>
<td>Doxorubicin, 1 d (n = 5)</td>
</tr>
<tr>
<td>Doxorubicin, 5 d (n = 8)</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
</tr>
<tr>
<td>292 ± 31</td>
</tr>
<tr>
<td>269 ± 13</td>
</tr>
<tr>
<td>224 ± 31*</td>
</tr>
<tr>
<td>LV pressure (mm Hg)</td>
</tr>
<tr>
<td>95 ± 6</td>
</tr>
<tr>
<td>75 ± 5</td>
</tr>
<tr>
<td>67 ± 6†</td>
</tr>
<tr>
<td>dp/dt max (mm Hg/s)</td>
</tr>
<tr>
<td>5,852 ± 1019</td>
</tr>
<tr>
<td>4,080 ± 381*</td>
</tr>
<tr>
<td>3,324 ± 604*</td>
</tr>
<tr>
<td>Stroke volume (μL)</td>
</tr>
<tr>
<td>36 ± 9</td>
</tr>
<tr>
<td>28 ± 6</td>
</tr>
<tr>
<td>19 ± 5†</td>
</tr>
<tr>
<td>Cardiac output (ml/min)</td>
</tr>
<tr>
<td>10.5 ± 1.9</td>
</tr>
<tr>
<td>7.7 ± 1.7</td>
</tr>
<tr>
<td>4.1 ± 0.9†</td>
</tr>
<tr>
<td>Ejection fraction (%)</td>
</tr>
<tr>
<td>58 ± 10</td>
</tr>
<tr>
<td>49 ± 6</td>
</tr>
<tr>
<td>46 ± 11*</td>
</tr>
<tr>
<td>dp/dt min (mm Hg/s)</td>
</tr>
<tr>
<td>−4,871 ± 668</td>
</tr>
<tr>
<td>−3,832 ± 341*</td>
</tr>
<tr>
<td>−2,979 ± 417†</td>
</tr>
<tr>
<td>Tau (ms)</td>
</tr>
<tr>
<td>11 ± 2</td>
</tr>
<tr>
<td>15 ± 2</td>
</tr>
<tr>
<td>16 ± 2*</td>
</tr>
<tr>
<td>Endarterial elastance (mm Hg/μL)</td>
</tr>
<tr>
<td>2.5 ± 0.7</td>
</tr>
<tr>
<td>2.75 ± 0.6</td>
</tr>
<tr>
<td>3.8 ± 1.2*</td>
</tr>
<tr>
<td>Stiffness index (mm Hg/μL)</td>
</tr>
<tr>
<td>0.03 ± 0.011</td>
</tr>
<tr>
<td>0.044 ± 0.015</td>
</tr>
<tr>
<td>0.138 ± 0.046†</td>
</tr>
</tbody>
</table>

*P < 0.05. †P < 0.005 versus control group.
Effect of bosentan on cardiac function in doxorubicin-treated animals. The influence of bosentan pretreatment on cardiac function is shown in Fig. 4. Administration of bosentan to control animals had no significant effect on cardiac function. Doxorubicin + bosentan–treated mice showed improved systolic (dp/dt\(_{max}\), +56%; LV pressure, +18%), diastolic (dp/dt\(_{min}\), /C0 47%), and global LV function as compared with doxorubicin-treated animals. Myocardial stiffness was significantly decreased (61%) in bosentan-treated doxorubicin-mice compared with doxorubicin-treated mice.

Effect of bosentan on cardiac function in doxorubicin-treated animals. The influence of bosentan pretreatment on cardiac function is shown in Fig. 4. Administration of bosentan to control animals had no significant effect on cardiac function. Doxorubicin + bosentan–treated mice showed improved systolic (dp/dt\(_{max}\), +56%; LV pressure, +18%), diastolic (dp/dt\(_{min}\), /C0 47%), and global LV function as compared with doxorubicin-treated animals. Myocardial stiffness was significantly decreased (61%) in bosentan-treated doxorubicin-mice compared with doxorubicin-treated mice.

Effect of bosentan on doxorubicin-induced TNF\(\alpha\) content, expression of Bax, GATA-4, and ET-1 as well as lipid peroxidation. Five days after doxorubicin administration, the TNF\(\alpha\) content was significantly increased from 5.3 (4.3–7.9; control) to 11.9 (7.8–20.1) pg/mg (Fig. 5A). This doxorubicin-mediated increase in cardiac TNF\(\alpha\) level was reduced by bosentan pretreatment to 7.9 (5.6–9.7) pg/mg, which was not significantly different from the control level. TNF\(\alpha\) itself is able to induce a variety of proapoptotic proteins such as Bax (12). Doxorubicin treatment of C57Bl/10 mice resulted in an enhanced Bax expression with an increase in arbitrary units from 0.72 (0.48–1.49) in control animals to 1.64 (1.36–1.91; \(P < 0.01\)) 5 days after doxorubicin application and unchanged Bcl-\(\text{\textunderscore}x\)\(\text{\textunderscore}L\) protein expression in the LV as determined by immunoblotting (Fig. 5B). Again, bosentan pretreatment caused a strong decline in Bax protein level to a value of 1.01 (0.77–1.2; \(P < 0.01\)). Furthermore, we tested whether doxorubicin treatment caused an increase of lipid peroxidation in LV by measurement of MDA/HAE levels. Five days after doxorubicin administration, there was a significant increase in MDA/HAE content from 746 (656–885) to 1,062 (1,002–1,188) nmol/L, which was significantly diminished by bosentan application to 874 (816–944) nmol/L (Fig. 5A).

It has been reported that doxorubicin reduces the expression of the transcription factor GATA-4 (13). Therefore, we also examined whether this effect was modulated by bosentan in vivo. As shown in Fig. 5C, 5 days after doxorubicin application, the GATA-4 mRNA content in the LV of mice was significantly reduced from...
0.83 (0.67–1.17) in control animals to 0.23 (0.19–0.5). Pretreatment of animals with bosentan resulted in an increased GATA-4 expression with a value of 1.47 (0.76–1.80). Five days after doxorubicin administration, we could also show a significant suppression of GATA-4 protein levels [arbitrary units of 1.10 (0.40–1.12) to 0.38 (0.09–0.78); \( P < 0.05 \)]. The decrease in GATA-4 protein was partly reversed by bosentan pretreatment [arbitrary units of 0.71 (0.24–1.09)] without reaching significance against doxorubicin treatment \( (P = 0.15; \text{Fig. } 5D) \).

In addition, we investigated whether bosentan alone affects ET-1 expression in the LV of C57Bl/10 mice. As shown in Fig. 5C, bosentan had no significant influence on ET-1 peptide expression of doxorubicin-treated animals. Moreover, ppET-1 mRNA was not altered by bosentan (data not shown).

**Discussion**

The clinical use of doxorubicin is limited by cardiotoxicity, which often leads to progressive heart failure with impaired contractility, arrhythmias, or sudden death (1). The precise mechanisms underlying this phenomenon have not been fully understood thus far. Therefore, characterization of these mechanisms and subsequent development of cardioprotective therapeutic strategies are of interest.

In the present study, a mouse model (C57Bl/10) of doxorubicin-induced cardiomyopathy was established and cardiac function was assessed using the microconductance pressure catheter method. We identified serious systolic and diastolic LV dysfunction with reduced LV pressure, \( dP/dt \), and cardiac output after doxorubicin application. In humans, cardiac failure is similarly characterized by LV dysfunction with impaired systolic and diastolic function, and a reduced LV ejection fraction is a commonly used parameter for doxorubicin-induced cardiomyopathy (14). These data suggest that our animal model resembles myocardial changes due to doxorubicin toxicity in patients.

Similar characteristics of cardiomyopathy such as significantly lower LV pressure and \( dP/dt_{\text{max}} \) were also found in mice overexpressing ET-1 orthotopically in cardiomyocytes (4). This led to the hypothesis that ET-1 might be an important mediator of doxorubicin cardiotoxicity. Accordingly, increased ppET-1 mRNA and ET-1 peptide contents were measured in the LV of C57Bl/10 mouse hearts after doxorubicin application in our study. ET-1 displays its effects through binding on ET\(_A\) or ET\(_B\) receptors. Both ET\(_A\) and ET\(_B\) receptors are present on cardiomyocytes and cardiac fibroblasts, with ET\(_A\) representing \( \sim 90\% \) of the endothelin receptors on cardiomyocytes (15). Besides increased expression of ET-1, its receptors are also elevated in the myocardium of rats with CHF and in patients with idiopathic dilated cardiomyopathy (5, 6). Such an elevation of endothelin receptor subtype A and B was not observed in the LV of mouse hearts after doxorubicin application in our study (data not shown). In order to elucidate the pathways used for the regulation of ET-1 by doxorubicin, signaling experiments were carried out in cell lines and adult rat cardiomyocytes. Investigations in the murine cardiomyocyte cell line HL-1 retaining differentiated cardiac morphologic, biochemical, and electrophysiological properties (16) confirmed the regulation of ET-1 by doxorubicin. In the human tumor cell line HeLa, a cellular system in which production, secretion, and function of ET-1 have been recently characterized in detail (17), doxorubicin also increased ET-1 expression. In HeLa cells, the up-regulation of ET-1 was caused by other anthracycline derivatives as well, including daunorubicin, epirubicin, and idarubicin, whereas cytostatics from other classes failed to increase ET-1 mRNA with the exception of mitoxantrone for which cardiotoxic events have been described as well (data not shown; ref. 18). Accordingly, treatment of adult rat ventricular cardiomyocytes with doxorubicin resulted in significant increases of ET-1 mRNA and peptide. In all investigated cells, the increase of ppET-1 mRNA was stronger compared with ET-1 peptide. This phenomenon might be caused by the fact that ET-1 is secreted, and therefore, the intracellular peptide content reflects only a fraction of the synthesized ET-1 (19).

Recently, it was shown that doxorubicin can activate the MEK1/2-ERK1/2 cascade due to ligand-independent activation of the EGF receptor (20). Both EGF receptor and MEK1/2-ERK1/2 were activated following doxorubicin administration in our study in vitro and in vivo in the LV of mouse hearts. Further inhibition experiments with the EGF receptor tyrosine kinase inhibitor AG1478 and the MEK1/2 inhibitor PD98059 confirmed the role of EGF receptor and MEK1/2 in the regulation of ET-1 expression. In the 5'-region of the ET-1 gene, several regulatory elements were located (21). Analysis of the ET-1 promoter showed that a potential regulatory site for ET-1 induction by doxorubicin is located between –68 and –517 bp upstream of the start ATG. In this region, an activator protein binding site is present at –109 bp (22), which is involved in ET-1 regulation by doxorubicin as shown in reporter gene assays with a mutated AP-1 binding site (data not shown).

Up to this point, we could show that ET-1 is modulated by doxorubicin in both animal and cellular models. In order to elucidate whether ET-1 plays a role in doxorubicin-induced cardiotoxicity, we investigated the influence of the orally administered combined ET\(_A/B\) receptor antagonist bosentan (23) on doxorubicin-induced cardiomyopathy. Pretreatment with bosentan before doxorubicin application significantly protected against doxorubicin-induced LV dysfunction (without having influenced the ET-1 level itself) as shown by the improved systolic and diastolic function compared with doxorubicin-treated animals. These effects are similar to those described with the endothelin receptor antagonist LU420627 in animals overexpressing ET-1 in cardiomyocytes (4). Aside from doxorubicin-induced cardiomyopathy, the endothelin system is activated in patients with chronic heart failure. In fact, the increased plasma ET-1 concentrations correlate with the hemodynamic severity and prognosis in these patients. Therefore, clinical trials of endothelin blockade in heart failure patients were undertaken. However, the results of these studies were neutral in terms of mortality and symptoms (24), leading to an intense discussion on whether the use of receptor subtype-specific endothelin receptor blockers may be of advantage. Interestingly, the above cited study by Yang and coworkers showed that the combined ET\(_A/ET_B\) antagonist LU420627, but not the selective ET\(_A\) antagonist LU135252, prolonged the survival of ET–1 overexpressing cardiomyopathic mice (4).

Moreover, ET-1 has been associated with cell growth, angiogenesis, and development of metastasis, and hence, to the progression of some types of cancer (e.g., prostate carcinoma, Kaposi sarcoma, and ovarian and breast cancer). Therefore, the use of endothelin receptor antagonists, including bosentan, as anticancer drugs has been addressed in experimental and clinical settings (25–28).

It is known that myocardial overexpression of ET-1 initiates an inflammatory cascade and elevates the expression of TNF\(_\alpha\), IFN-\(\gamma\), interleukin 1, and interleukin 6. We therefore investigated the effect of doxorubicin on the production of the proinflammatory cytokine TNF\(_\alpha\). Five days after doxorubicin application, we detected an
increase in TNF-\alpha content in the LV of mouse hearts, which is in accordance with a previous study of Nozaki et al. (7). TNF-\alpha itself is cardiotoxic and induces the depression of cardiac function (29). Moreover, a cardiac-specific overexpression of TNF-\alpha in transgenic mice leads to cardiomyopathy (30). Whether TNF-\alpha is a mediator of doxorubicin-induced cardiomyopathy has not been clarified thus far. However, when mice were treated with doxorubicin plus bosentan, the induction of TNF-\alpha was significantly reduced, which implicates that ET-1 may play a role in the regulation of TNF-\alpha expression and that both are involved in doxorubicin-induced cardiotoxicity. Such an inflammatory component in doxorubicin-mediated cardiotoxicity was already suggested in 1993 by Gaudin and coworkers (31) and is underlined by a study of Deepa and Varalakshmi in which inflammatory changes in the cardiac tissue of rats given doxorubicin were observed (32). Furthermore, the role of inflammation is corroborated by the recently published data that doxorubicin strongly induced cyclooxygenase-2 expression, which was reduced by erythropoietin following the prevention of cardiomyopathy (33).

A potential role of apoptotic processes in the development of heart failure is discussed. Apoptosis may contribute to cardiomyocyte loss and structural changes accompanied by up-regulation of both proapoptotic and antiapoptotic Bcl-2 family member Bax (34). Accordingly, we found a doxorubicin-mediated increase in the cardiac protein expression of Bax which was reduced under bosentan treatment. It is possible that increased Bax expression is induced by TNF-\alpha, for which the stimulation of proapoptotic factors was shown (35). Kim et al. could show that doxorubicin suppressed the expression of GATA-4 and suggest an antiapoptotic role of this transcription factor in cardiomyocytes, particularly by the regulation of Bel-2 (13). Suppression of GATA-4 mRNA in the LV of mouse hearts was confirmed by our study, supporting the initiation of apoptotic processes. Moreover, pretreatment with bosentan restored the GATA-4 mRNA to the control level, again indicating prevention against apoptotic processes.

The generation of ROS and lipid peroxidation have also been discussed as a factor contributing to doxorubicin cardiotoxicity (36, 37). In our mouse model, bosentan reduced the doxorubicin-induced lipid peroxidation indicating the participation of ET-1 in lipid peroxidation. A possible source of the increased ROS leading to lipid peroxidation could be the enzyme NADP(H) oxidase. In smooth muscle cells and blood vessels, ET-1 activates NADP(H) oxidase (38, 39), suggesting a possible activation in the heart as well. This finding is supported by recently discovered polymorphisms of the NADP(H) oxidase which are associated with anthracyclin-induced cardiotoxicity (40).

In summary, our study shows that doxorubicin mediates the up-regulation of ET-1 expression which is accompanied by LV dysfunction resembling doxorubicin-induced cardiomyopathy in patients. Doxorubicin-modulated ET-1 expression involves the activation of the EGF receptor and the MEK1/2–ERK1/2 cascade. Finally, the combined endothelin receptor antagonist bosentan reduced doxorubicin-mediated LV dysfunction accompanied with decreased TNF-\alpha and Bax, diminished lipid peroxidation, and increased GATA-4 expression. Aside from mechanistic aspects, the present investigations suggest the cardioprotective role of bosentan in doxorubicin-induced cardiomyopathy, which may be of therapeutic benefit for patients receiving doxorubicin.

Acknowledgments

Received 4/11/2007; revised 7/11/2007; accepted 8/21/2007. Grant support: Deutsche Forschungsgemeinschaft SFB/TR 19-04. R. Ewert and H.K. Kroemer received consulting fees for scientific lectures from Actelion Pharmaceuticals Deutschland GmbH (Freiburg, Germany), the manufacturer of Bosentan.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We are grateful to Dan M. Roden (MD, Vanderbilt University, Nashville, TN) for helpful comments, Kerstin Boettcher (Department of Pharmacology, Greifswald, Germany) for her excellent technical assistance, and Danilo Wegner for his guidance in statistical analysis.

References

10. Piper HM, Probst I, Schwartz P, et al. Culturing of vascular smooth muscle cells and blood vessels, ET-1 activates NADP(H) oxidase. In inflammatory changes in the cardiac tissue of rats given doxorubicin are corroborated by the recently published data that doxorubicin strongly induced cyclooxygenase-2 expression, which was reduced by erythropoietin following the prevention of cardiomyopathy (33).

Cancer Research 2007; 67: (21). November 1, 2007 10434 www.aacrjournals.org

© 2007 American Association for Cancer Research.
The Endothelin Receptor Blocker Bosentan Inhibits Doxorubicin-Induced Cardiomyopathy


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/67/21/10428

Cited articles
This article cites 40 articles, 20 of which you can access for free at:
http://cancerres.aacrjournals.org/content/67/21/10428.full.html#ref-list-1

Citing articles
This article has been cited by 11 HighWire-hosted articles. Access the articles at:
/content/67/21/10428.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.