Original Article

Angiotensin II Type 1a Receptor Mediates Doxorubicin-Induced Cardiomyopathy

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Although the serious cardiotoxicity of doxorubicin (DOX), a useful chemotherapeutic agent, limits the use of this agent, the mechanism of DOX-induced cardiomyopathy remains unclear. Since accumulating evidence suggests that activation of the renin-angiotensin system is involved in the development of various types of cardiovascular remodeling, we examined the role of angiotensin II (Ang II) in DOX-induced cardiotoxicity using Ang II type 1a receptor (AT1) knockout (KO) mice. To examine the role of AT1 in the acute effects of DOX, we injected a single 20 mg/kg dose of DOX into AT1KO mice, wild type (WT) mice and WT mice treated with an AT1 antagonist, RNH-6270; to examine the role of AT1 in the chronic effects of DOX, we injected mice of the same groups with 1 mg/kg DOX once a week for 12 weeks. Echocardiography revealed that cardiac function was significantly impaired in WT mice, but not in AT1KO mice or WT mice administered RNH-6270, by both acute and chronic DOX treatment. Histological analysis showed that DOX induced myofibrillar loss and increased the number of apoptotic cells in WT mice, but not in AT1KO mice or WT mice administered RNH-6270. Expression of the ANP gene was downregulated by DOX treatment in WT mice, and this alteration was attenuated in AT1KO mice and in RNH-6270-treated mice. We conclude that the AT1-mediated Ang II signaling pathway plays an important role in DOX-induced cardiac impairment, suggesting that an AT1 antagonist can be used to prevent DOX-induced cardiomyopathy. (Hypertens Res 2002; 25: 597–603)

Key Words: angiotensin II, angiotensin II type 1 receptor antagonist, cardiac remodeling, doxorubicin, knockout mouse

Introduction

Anthracyclines such as doxorubicin (DOX) and daunomycin are useful chemotherapeutic agents for hematological cancers, carcinomas and sarcomas. However, anthracyclines frequently induce irreversible and life-threatening cardiac dysfunction, and this cardiac toxicity limits their clinical use (1). The clinical features of the cardiotoxicity induced by anthracyclines can be divided into acute and chronic types (2). Acute injuries occur immediately after treatment and may cause transient arrhythmia, pericarditis, myocarditis, and acute failure of the left ventricle. Chronic effects of anthracyclines depend on the cumulative dose and result in dilated cardiomyopathy-like congestive heart failure (3). Although several mechanisms, such as free radical-dependent lipid peroxidation (4), mitochondria impairment, and modification of cardiac calcium transport (5), have been reported to be a cause of the cardiotoxicity of anthracyclines, the precise mechanism of myocardial impairment remains unclear.
Many lines of evidence have suggested that the renin-angiotensin system (RAS) plays an important role in the development of cardiac hypertrophy, failure and reperfusion injury (6). Suppression of the RAS ameliorates the remodeling process of the heart and prolongs long-term survival in animal models and humans with cardiac hypertrophy, failure and reperfusion injury (7–12). We have reported that reperfusion arrhythmia and left ventricular (LV) remodeling after myocardial infarction are reduced in angiotensin II (Ang II) type 1a receptor (AT1) knockout (KO) mice (13–15). These results and observations suggest that the RAS is critically involved in the development of various cardiac abnormalities.

In the present study, we examined whether Ang II was also involved in DOX-induced cardiotoxicity. We injected DOX into AT1KO mice and wild type (WT) mice and examined cardiac function, morphological features, apoptosis and gene expressions. In addition, because we and others have previously reported that blood pressure was lower in AT1KO mice than WT mice (14, 15), we suspected that blood pressure might be important for DOX-induced cardiotoxicity. Therefore, we also examined the DOX-induced cardiac effects in WT mice treated with an AT1 antagonist, RNH-6270 (16), but RNH-6270 had no affect on blood pressure at the dose used in this study.

Methods

Animals

Eight-week-old male WT mice \((n = 86)\) and AT1KO mice \((n = 24)\) from the same genetic background were used in the present study. Mice were housed under climate-controlled conditions with a 12-h light/dark cycle and were provided with standard food and water ad libitum as described previously (7). All protocols were approved by the Institutional Animal Care and Use Committee of the University of Chiba.

DOX Injection

WT mice were divided into three groups: a control group \((n = 12)\); a group of DOX (Kyowa Hakko Co. Ltd., Tokyo, Japan) -injected WT mice \((n = 12)\), and a group of mice treated with RNH-6270 1 day before receiving DOX \((n = 12)\). AT1KO mice were also divided into two groups: a control group \((n = 12)\) and a DOX-treated group \((n = 12)\). RNH-6270 (Sankyo Co. Ltd., Tokyo, Japan) was continuously infused at 1 mg/kg/day with an osmotic minipump (Alza Co., Palo Alto, USA).

Recently, myocardial apoptosis has been reported to be a cause of heart failure (17, 18), and we have reported that treatment of DOX induced apoptosis of cardiomyocytes in vitro (19). In the present study, we therefore used DOX to induce heart failure. We first performed a pilot study with WT mice \((n = 54)\). The mice were given 10, 20 or 30 mg/kg of DOX and sacrificed at 1, 2, 4, 12, 24 or 48 h after DOX injection. The maximum cardiomyocyte apoptosis was achieved by a dose of 20 mg/kg DOX at 24 h after injection, so we used a dose of 20 mg/kg and time of 24 h for the acute study. To further examine the role of the RAS in DOX-induced cardiotoxicity, we observed the survival rate of mice injected with 20 mg/kg for 4 weeks.

For the chronic study, we selected the protocol of 1 mg/kg/day of DOX injected intraperitoneally once a week for 12 weeks, because it has been reported that this protocol in mice corresponds to several months of DOX treatment in humans (20). The mice were sacrificed and examined at the 13th week.

Physiological Studies

Mice were weakly anesthetized by intraperitoneal injection of 50 mg/kg of ketamine HCl (Sigma Chemical Co., St. Louis, USA) and 2.5 mg/kg of xylazine (Sigma Chemical Co.). Transthoracic echocardiographic analysis was performed using an HP sonos 100CF (Hewlett-Packard, Palo Alto, USA) as described previously (14). LV internal dimensions, such as end-diastolic dimension (LVEDd) and end-systolic dimension (LVEDs), were measured, and the percent fractional shortening (%FS) was calculated as \(\left\{\frac{\text{LVEDd} - \text{LVEDs}}{\text{LVEDd}}\right\} \times 100\).

Histological Analysis

The specimens were fixed with phosphate-buffered 10% formaldehyde for 24 h and then embedded in paraffin for light microscopic analysis. Four micrometer-thick paraffin sections were stained with hematoxylin-eosin and Azan. For the detection of apoptotic cells, TUNEL and immunohistochemical analysis to detect active caspase-3 were performed using an in situ apoptosis detection kit (Takara Syuzo, Kyoto, Japan) and anti-active caspase-3 polyclonal antibody (Promega, Madison, USA), respectively, according to the supplier’s instructions. For electron microscopic analysis, the specimens were fixed in 4% paraformaldehyde (Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing 0.25% glutaraldehyde (Sigma Chemical Co.), postfixed in 1% osmium tetroxide (MERCK & Co. Inc., Whitehouse Station, USA) and embedded in Epon 812 (TAAB Laboratories Equipment Ltd., Berkshire, UK). Ultrathin sections were stained with uranyl acetate (MERCK & Co. Inc.) and lead citrate (Nacalai Tesque, Kyoto, Japan). All samples were coded and scored in a blind fashion as described previously (21). We examined five hearts from each group, and ten pictures were taken of each heart. Samples were coded and scored independently by two people (H.T. and M.S.) using a scale of 0–4.
Northern Blot Analysis

Total RNA was extracted from left ventricles with RNAzol B (Biotecx Laboratories, Friendswood, USA). Ten micrograms of total RNA were separated on a 1.2% agarose-formaldehyde gel and blotted onto a Hybond-N membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). Partial cDNAs of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), cardiac ankyrin repeat protein (CARP) and myosin light chain 2v (MLC2v) were labeled with α-32 PdCTP (Amersham Pharmacia Biotech) using a random primer labeling kit (Takara Shuzo).

Statistical Analysis

Data are shown as the mean ± SEM. Multiple group comparison was performed by one-way ANOVA followed by the Bonferroni procedure for comparison of means. Values of p < 0.05 were considered to indicate statistical significance.

Table 1. Cardiac Geometry after DOX Injection

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<thead>
<tr>
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<th>WT</th>
<th>AT1KO</th>
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<tr>
<td></td>
<td>Control</td>
<td>DOX-injected</td>
</tr>
<tr>
<td>Acute</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVEDd (mm)</td>
<td>3.82 ± 0.08</td>
<td>4.31 ± 0.25</td>
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<tr>
<td>LVEDs (mm)</td>
<td>2.10 ± 0.04</td>
<td>2.60 ± 0.25*</td>
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<tr>
<td>Chronic</td>
<td></td>
<td></td>
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<tr>
<td>LVEDd (mm)</td>
<td>4.01 ± 0.03</td>
<td>4.35 ± 0.08*</td>
</tr>
<tr>
<td>LVEDs (mm)</td>
<td>2.25 ± 0.10</td>
<td>2.59 ± 0.11*</td>
</tr>
</tbody>
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Values are mean ± SEM. *p < 0.05, vs. control of same strain. LVEDd, left ventricular end-diastolic dimension; LVEDs, left ventricular end-systolic dimension.

Fig. 1. Cardiac geometry and function after DOX injection. In WT mice, %FS was decreased in both acute (A) and chronic (B) treatments. Cardiac functions were not changed in either WT mice treated with RNH-6270 or AT1KO mice. *p < 0.01, †p < 0.05. n.s., not significant.

Results

DOX Impaired the Cardiac Function of WT Mice but Not of AT1KO Mice or WT Mice Administered RNH-6270

Echocardiographic analysis was used to examine the effects of DOX on the LV geometry and function. The results showed that LV internal dimensions were significantly enlarged in WT mice by both acute (LVEDd 2.10 ± 0.04 vs. 2.60 ± 0.25, p < 0.05) and chronic DOX treatment (LVEDs 2.25 ± 0.10 vs. 2.59 ± 0.11; LVEDd 4.01 ± 0.03 vs. 4.35 ± 0.08, p < 0.05) (Table 1). %FS was concomitantly decreased in WT mice by both acute (45.1 ± 1.4 vs. 40.6 ± 0.8, p < 0.01) (Fig. 1A) and chronic DOX treatment (44.2 ± 2.1 vs. 37.9 ± 1.3, p < 0.05) (Fig. 1B). LV internal dimensions (Table 1) and cardiac function (Fig. 1A, B) were not changed by DOX treatment in WT mice treated with RNH-6270 or in AT1KO mice. These results suggest that blocking of the AT1-mediated signals rescues the heart from DOX-induced cardiotoxicity.

In addition, to examine the role of the RAS in DOX-induced cardiotoxicity over a longer period of time, we exam-
Fig. 2. A, Electron microscopic analysis. DOX induced morphological changes, such as cytoplasmic vacuolization and loss of myofilaments (arrows), in WT mice, but not in WT mice treated with RNH-6270 or AT1KO mice. Bar = 1µm. B: Electron microscopic score. DOX induced an increase of electron microscopic scores in WT mice, but not in WT mice treated with RNH-6270 or AT1KO mice in either the acute or chronic treatment groups. * p < 0.05.

ined the long-term survival after injection of 20 mg/kg of DOX. We found that none of the WT mice, AT1KO mice or WT mice treated with RNH-6270 had died at 4 weeks after DOX injection. This finding would suggest that the mild DOX-treatment protocol employed in this study causes no death in these animal models.

DOX Induced Cytoplasmic Vacuolization and Myofibrillar Loss in Cardiomyocytes of WT Mice but Not in Those of AT1KO Mice or WT Mice Administered RNH-6270

To elucidate the reason for the differences in cardiac geometry and function after DOX treatment between WT mice and AT1KO mice, we performed histological analysis using light and electron microscopy. The light microscopic analysis revealed that there were no morphological changes—including changes in cardiomyocyte diameter and interstitial and perivascular fibrosis—in any of the groups (data not shown). Electron microscopic analysis revealed that in WT mice, acute and chronic treatment of DOX induced significant changes in cardiomyocytes, such as cytoplasmic vacuolization and myofibrillar loss, while there were no ultrastructural changes in myocytes of WT mice treated with RNH-6270 or those of AT1KO mice after DOX injections (Fig. 2A). Electron microscopic scores were also increased only in WT mice injected with DOX for both acute and chronic studies (Fig. 2B). These results suggest that Ang II is involved in DOX-induced myocardial injury.

DOX Increased the Number of Apoptotic Cells

We have reported that daunomycin, one of the anthracyclines, induced apoptosis of cultured cardiac myocytes (19). To examine whether DOX also induces apoptosis of cardiac myocytes in vivo, we performed TUNEL analysis and immunohistochemical analysis to detect active caspase-3, a critical protease for apoptosis (22).

In the acute study, DOX significantly increased the number of TUNEL-positive cells in the hearts of WT mice (3.63 ± 0.57 cells out of 10,000 cardiomyocytes vs. control, 0.30 ± 0.06 cells, p < 0.01), but not in WT mice treated with RNH-6270 (0.50 ± 0.15 cells) or AT1KO mice (0.90 ± 0.21 cells vs. control, 0.20 ± 0.06 cells) (Fig. 3A). In the chronic phase, there were no significant differences in the number of TUNEL-positive cells in all groups (Fig. 3B). Immunohistochemical analysis showed that DOX significantly increased the number of active caspase-3-positive cells in the hearts of WT mice after both acute and chronic treatment (acute, 1.22 ± 0.22 cells out of 10,000 cardiomyocytes vs. no detectable cells in controls, p < 0.01; chronic, 1.11 ± 0.11 cells vs. 0.08 ± 0.08 cells in the controls, p < 0.01) (Fig. 3C, D). But active caspase-3-positive cells were not detected in either WT mice treated with RNH-6270 or AT1KO mice. These re-
results suggest that DOX induces apoptotic cell death of cardiac myocytes at least in part through AT1.

DOX Regulated Gene Expressions in the Heart

It has been reported that gene expressions of ANP, BNP, CARP and MLC2v are influenced by anthracyclines both in vivo and in vitro (23–27). To clarify the role of Ang II in DOX-induced gene expressions, we examined the mRNA levels of ANP, BNP, CARP and MLC2v by Northern blot analysis. In the acute phase, DOX strongly downregulated ANP, BNP and CARP genes in WT mice (Fig. 4). Treatment with RNH-6270 significantly attenuated suppression of the ANP gene but did not attenuate suppression of the BNP or CARP genes. The expression of the ANP gene was not downregulated in AT1KO mice (Fig. 4). These results suggest that the DOX-induced change of ANP gene expression is dependent at least in part on Ang II. On the other hand, there was no significant change in mRNA levels in either WT or AT1KO mice by chronic treatment with DOX (data not shown).

Discussion

Cardiotoxicity is a serious side effect of DOX; however, the precise mechanisms underlying the cardiac impairment by DOX remain unclear. In this study, we examined the role of Ang II in DOX-induced cardiotoxicity using AT1KO mice and an AT1 antagonist, RNH-6270. Cardiac dysfunction and ultrastructural changes were induced by both acute and chronic treatment of DOX, while only acute treatment resulted in changes in the expression of genes and an increase in the number of apoptotic cells. All these changes after either acute or chronic treatment with DOX were attenuated in AT1KO mice and by RNH-6270 treatment. Although the mechanism of cardiac dysfunction may be different between the acute and chronic phases, these results suggest that AT1-mediated Ang II signals are at least partially involved in DOX-induced cardiac injuries.

Accumulating evidence suggests that activation of the RAS is involved in the development of various cardiovascular remodeling (6–14). We have demonstrated the impor-
tance of RAS in LV remodeling after myocardial infarction using AT1KO mice (14). Reperfusion-induced arrhythmia is also significantly reduced in AT1KO mice (13), indicating that RAS plays an important role in various types of cardiac remodeling. In the present study, DOX induced LV remodeling, including LV dilatation and cardiac dysfunction, in WT mice, but not in AT1KO mice or WT mice administered RNH-6270, suggesting that DOX-evoked cardiac remodeling is also mediated by AT1.

DOX-induced changes in cardiac function and geometry were modest and no morphological changes were observed by light microscopy. Although some studies have reported that DOX treatment induces significant morphological changes (28), others have reported that there is no significant morphological change until 5 days after acute treatment (25, 27, 29) or until 8 weeks after the beginning of chronic treatment (30). In the present study, we used a mild treatment protocol that corresponded to a treatment in humans, and this may have been the reason for the modest DOX-induced changes.

We also investigated whether the hemodynamic effects affected the cardioprotective effects of RNH-6270. Because it has been reported that the blood pressure of AT1KO mice was lower than that of WT mice (15), we considered that lower blood pressure might have played a role in the absence of DOX-induced cardiac damage in AT1KO mice. However, we found no significant reduction of blood pressure in the mice treated with RNH-6270 (data not shown), which is compatible with previous reports indicating that a low dose of RNH-6270 (0.5–1 mg/kg/day) does not decrease blood pressure in various animal models (31–33). These results suggest that inhibition of the RAS in the heart may attenuate DOX-induced cardiac damage via a mechanism independent of blood pressure.

Acute treatment with DOX increased the number of TUNEL-positive cells and anti-active caspase-3 antibody-positive cells. These results suggest that DOX induced apoptotic death of cardiac myocytes, at least in the acute phase. Chronic treatment with DOX increased the number of cells positive for anti-active caspase-3 antibody, but not the number of TUNEL-positive cells. The reason for this discrepancy is not clear at present, but there are several possibilities. Although activation of caspase-3 is important for the induction of apoptosis, cells positive for anti-active caspase-3 antibody may not always undergo apoptosis. Since apoptotic cells usually disappear within a short period, a small increase in TUNEL-positive cells might not be recognized. In addition, there may be differences in sensitivity between the TUNEL method and the active caspase-3 detection system.

Plasma ANP levels are usually increased in various cardiovascular diseases, such as cardiac hypertrophy and heart failure. It has been reported, however, that plasma levels, mRNA levels and the promoter activity of the ANP gene were all suppressed by DOX treatment at the acute phase (23, 24), and that these changes may result from the production of ROS. ANP production is increased as an adaptational mechanism to decrease volume overload and wall stress on the myocardium. Therefore, it is a serious problem that DOX treatment downregulates ANP in spite of impaired cardiac function, making it even more important to protect the heart from DOX-induced cardiotoxicity. In the present study, acute DOX treatment decreased ANP mRNA levels in WT mice but not in AT1KO mice or WT mice treated with RNH-6270. These results suggest that blocking AT1 is effective to prevent DOX-induced downregulation of ANP as well as DOX-induced cardiomyocyte injury and death.

On the other hand, the ANP mRNA level was not changed by DOX treatment in our chronic trial. In a previous study, ANP mRNA levels were decreased 4 days after DOX treatment, but returned to the control levels after 4 weeks (25). We therefore consider that ANP mRNA levels depend on the balance between direct effects of DOX that suppress ANP gene expression and indirect effects of DOX that increase ANP mRNA levels by reducing cardiac function.

References


33. Koike H: New pharmacologic aspects of CS-866, the newest angiotensin II receptor antagonist. *Am J Cardiol* 2001; 87: 33C–36C.