Telmisartan inhibits the progression of cardiomyopathy in daunorubicin treated rats: the role of advanced glycation end products

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Abstract

Background: Anthracyclines have been reported to induce cardiotoxicity through mechanisms involving formation of advanced glycation end-products (AGEs), including pentosidine and Nε-(carboxymethyl)lysine (CML). We investigated the potential utility of telmisartan (TLM), an angiotensin II receptor antagonists (ARB) on anthracycline-induced cardiotoxicity.

Methods: Three groups of Sprague-Dawley rats were treated as follows: The first group received daunorubicin (DNR) 3 mg/kgBW every alternating day to reach a cumulative dose of 9 mg/kg DNR. The second group received DNR plus TLM at a dose of 10 mg/kgBW, by oral gavage for 6 weeks, and the third group served as control group (CTL) which only received vehicle of DNR. Mean blood pressure (MBP), peak left ventricular pressure (LVP), LV end-diastolic pressure (LVEDP), and intra-ventricular contractility (+dP/dt) were recorded by using Powerlab instrumentation. Ejection fraction (EF), and fractional shortening (FS) were measured by echocardiography. Expression of receptor of AGE (RAGE), pentosidine and CML were measured in LV tissue.

Results: DNR treatment was associated with significant weakening of some hemodynamic parameters which could be reversed by TLM (LVP: 124.3 ± 6.0; 111 ± 7; and 115.1 ± 5.4 mmHg, respectively in CTL, DNR, and DNR-TLM groups; LVEDP: 7.5 ± 0.9; 10.7 ± 0.3; 8.7 ± 0.4 mmHg, respectively; +dP/dt: 6813 ± 541; 4800 ± 345; 5950 ± 398 mmHg/s, respectively). The same phenomena were also observed on echocardiographic parameters (EF: 78.9 ± 1.8; 59.6 ± 1.4; 76.2 ± 2.7 %; FS: 42.8 ± 1.7; 29.1 ± 1.3; 41 ± 2.7 %, respectively). Expression of RAGE as well as pentosidine and CML were increased in DNR-rats. TML treatment ameliorated these changes.

Conclusion: These results suggested the role of AGE formation in DNR-induced cardiotoxicity and telmisartan could inhibit the progression of cardiac toxicity at least in part by reduction RAGE expression. (Med J Indones 2011; 20:255-62)

Keywords: advanced glycation end product, anthracycline, cardiotoxicity, daunorubicin, telmisartan

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Daunorubicin (DNR), one of anthracycline antibiotics are potent antineoplastic agents. Unfortunately, despite its broad effectiveness, the clinical use of DNR is limited by a dose dependent and cumulative cardiotoxicity. The adverse effect can vary from transient electrocardiography abnormalities to cardiomyopathy and heart failure. Among the proposed mechanisms by which anthracyline cause irreversible myocardial injury, free radical formation is generally accepted as the main mechanism. Cardiomyocytes have poor antioxidant defense systems, and free oxygen radicals can damage various targets in these cells.

Non-enzymatic modification of proteins by a formation of reduced-sugar leads to the formation of advanced glycation end products (AGEs), whose process has been reported to progress under physiological aging, oxidative stress or diabetic conditions. There have been only a few reports outlining the contribution of AGE formation to the adverse effects of medicinal drugs. Very recently, Moriyama et al reported that doxorubicin (DOX), another anthracycline, accelerated the formation of pentosidine and N-(carboxymethyl)lysine (CML), both of them are well known AGEs, in the heart through enhanced oxidative stress and its suggests that AGE formation is involved in DOX-induced cardiomyopathy.

Telmisartan is a selective angiotensin II (Ang II) type I receptor (AT1R) blocker (ARB) that is used in the hypertension treatment, and it exerts a variety of pleiotropic effects, including anti-oxidative, anti-apoptotic, and anti-inflammatory effects. Furthermore, telmisartan could confer effects other than the blockage of the AT1R, such as peroxisome proliferator-activated receptor (PPAR-γ) activation and there were evidences reported that telmisartan could down-regulate RAGE expression and suppress its downstream signaling in various cells through its unique PPAR-γ modulating activity. Previously, we have reported the effect of telmisartan in limiting the cardiotoxic effect of DNR by inhibiting the action of Ang II via AT-1R, which reverses oxidative stress and myocardial apoptosis, but this study was conducted for only 12 days, as acute model and did not investigate the involvement of AGES and RAGE in DNR-induced cardiomyopathy.

Based on those above reports, it is of interest to check whether there is an involvement of AGES and its receptor RAGE in DNR-induced cardiomyopathy in rats. In addition it was known that ARB and angiotensin-converting enzyme (ACE) inhibitor inhibit the formation of two AGES, pentosidine and CML and the expression of its receptor RAGE in vitro, indicating a cross-talk exists between the AGE–RAGE system and the renin angiotensin system (RAS). Taking all the above facts together, we examined here whether AGE and RAGE involves in DNR-induced cardiomyopathy and verify the efficacy of 6 weeks of treatment with telmisartan. We posited that telmisartan may inhibit the formation of AGE, expression of RAGE in association with attenuation of myocardial damage induced by DNR.

**METHODS**

This study was done between July to September 2010 Department Clinical Pharmacology Niigata University of pharmacy and applied life science and data analysis was performed at the Department of Pharmacology and Therapeutics. Faculty of Medicine, Universitas Indonesia.

**Drugs and chemicals**

Unless otherwise stated all reagents were of analytical grade and purchased from Sigma (Tokyo, Japan). DNR was kindly donated by Meiji Seika Kaisha Ltd., Tokyo, Japan. Telmisartan was donated by Boehringer Ingelheim GmbH (Ingelheim am Rhein, Germany).

**Animals and treatment**

Eight-week-old male Sprague-Dawley rats were obtained from Charles River Japan Inc. (Kanagawa, Japan). The animals were quarantined and acclimatized for 2 weeks prior to the initiation of the experiments. On day 0, each animal received a single intravenous injection of DNR at a dose of 3 mg/kg (i.v.). The drug was administered in three equal injections at 48 h intervals for a period of one week to achieve an accumulative dose of 9 mg/kg, which is well documented to produce cardiotoxicity. Age-matched rats were injected with corresponding volumes of 0.9% NaCl and used as a control (group CTL; n=5). Twenty-two DNR-treated rats were randomly divided into two groups and received oral administration of telmisartan (10 mg/kg/day; group DNR+TLM; n=10) or vehicle (group DNR; n=12). The dose of telmisartan was chosen on the basis of the previous reports. Administration of telmisartan was started on the same day as DNR administration and continued for 5 additional weeks after cessation of DNR administration (6 weeks total period). This duration of study was chosen on the basis of the previous reports. The animal experiments were performed in accordance with national guidelines for the use and care of laboratory animals and were approved by the local animal committee of Niigata University of Pharmacy and Applied Life Sciences.

**Hemodynamic and echocardiographic study**

After the end of the study period (6 weeks), rats were anesthetized with 2% halothane in O₂ and subjected
to surgical procedures to measure hemodynamic parameters. After the instrumentation, the concentration of halothane was reduced to 0.5% to record steady-state hemodynamic data. Hemodynamic parameters such as mean blood pressure (MBP), peak left ventricular pressure (LVP), LV end-diastolic pressure (LVEDP), and the rate of intra-ventricular pressure rise and decline (+dP/dt) were recorded as previously described.\textsuperscript{16}

Two-dimensional echocardiographic studies were performed under 0.5% halothane anesthesia using an echocardiographic machine equipped with a 7.5-MHz transducer (SSD-5500; Aloka, Tokyo, Japan). M-mode tracings were recorded from the epicardial surface of the right ventricle; the short axis view of the left ventricle was recorded to measure the LV dimension in diastole (LVDd) and LV dimension in systole (LVDs). LV fractional shortening (FS) and ejection fraction (EF) were calculated and expressed as percentages. The study was performed in a blinded manner.

**Histopathological analysis**

After the measurement of echocardiographic parameters, hearts were excised and weighed immediately (HW), and its ratio to BW (HW/BW) was calculated. Half of heart was immediately snap-frozen in liquid nitrogen for subsequent protein extraction and enzymatic assays. The remaining excised hearts were cut into about 2-mm-thick transverse slices and fixed in 10% formalin. After being embedded in paraffin, several transverse sections were obtained from the heart and stained with hematoxylin and eosin (H&E) for histological evaluation, and also stained with Azan-Mallory to demonstrate fibrosis in heart tissues. The frequency and the severity of lesions in the hearts were assessed semi-quantitatively as previously reported\textsuperscript{13,17} by light microscopy using a scale where score 0, normal; 1, mild; 2, moderate; and 3, severe. The scoring criteria for myocardial lesions included the degree of myocyte vacuolization with respect to size and the number of vacuoles, degree of fibrosis, myocardial degeneration and interstitial edema and loss of myofibrils.

**Immunohistochemistry for pentosidine and RAGE**

Formalin-fixed, paraffin-embedded heart tissue sections were used for immunohistochemical staining. After deparaffinization and hydration, the slides were washed in TBS and then horseradish peroxidase (HRP)-conjugated secondary antibody was added and the slides were further incubated at room temperature for 45 min. The slides were washed in TBS and incubated with diaminobenzidine tetrahydrochloride as the substrate, and counterstained with hematoxylin. A negative control without primary antibody was included in the experiment to verify the antibody specificity.

**Protein analysis by Western blotting**

Protein lysate was prepared from heart tissue as described previously.\textsuperscript{18} The total protein concentration in samples was measured by the bicinchoninic acid method.\textsuperscript{19} For the determination of protein levels of CML and RAGE, equal amounts of protein extracts (30 µg) were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (Bio-Rad, CA, U.S.A) and transferred electrophoretically to nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline Tween (20 mM Tris, pH 7.6, 137 mM NaCl, and 0.1% Tween 20). All antibodies were purchased from Santa Cruz Biotechnology Inc., CA, USA aside from NF-κB p65 (Cell Signaling Technology Inc., Beverly, MA, USA), and CML (Abcam Inc., Cambridge, UK) and used at a dilution of 1:1000. The membrane was incubated overnight at 4°C with the primary antibody, and the bound antibody was visualized using the respective HRP-conjugated secondary antibodies (Santa Cruz Biotechnology Inc.) and chemiluminescence developing agents (GE Healthcare, Buckinghamshire, UK). The level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was estimated in every sample to check for equal loading of samples. Films were scanned and band densities were quantified with densitometric analysis using Scion Image program (Epson GT-X700, Tokyo, Japan). Finally, Western blot data were normalized by those for heart GAPDH.

**Statistical analysis**

Data are presented as mean ± S.E.M and were analyzed using one-way analysis of variance followed by Tukey or Bonferroni methods for post-hoc analysis and two-tailed t-test when appropriate. A value of \( p < 0.05 \) was considered statistically significant. For statistical analysis GraphPad Prism 5 software (San Diego, CA, USA) was used.

**RESULTS**

Six of 12 rats in DNR group died between days 14 and 42 (Table 1). None of the rats died in group Control and DNR-TLM.

**Effect of telmisartan on myocardial functions**

LVEDP was significantly higher (10.7±0.3 vs 7.5±0.9 mmHg, \( p<0.05 \)) and LVP and \( +dP/dt \) were significantly...
lower in group DNR than in group Control (111±7 vs 124.3 mmHg, p<0.05; and 4800±345 vs 6813±541 mmHg/s, p<0.05; 4135±365 vs 7290±775 mmHg/s, p<0.05, respectively), indicating systolic and diastolic dysfunction in DNR rats. Telmisartan treatment improved the myocardial dysfunction by significant reduction in LVEDP (8.7±0.4 vs 10.7±0.3 mmHg, p<0.05) and elevation in the +dP/dt (5950±341 vs 4800±345 mmHg/s, p<0.05; 5950±398 vs 4135±365 mmHg/s, p<0.05, respectively) compared with those in group DNR (Table 1).

Echocardiographic data revealed that LV systolic function, as assessed by FS and EF was reduced significantly in group DNR compared with that in group Control (29.1±1.3 vs 42.8±1.7 %, p<0.05; and 59.6±1.4 vs 78.9±1.8 %, p<0.05, respectively). The reductions in both FS and EF were significantly attenuated in group DNR+Telm (41±2.7 vs 29.1±1.3 %, p<0.05; and 76.2±2.7 vs 59.6±1.4 %, p<0.05, respectively) (Table 1).

**Effect of telmisartan on cardiac histopathology**

Histological changes in heart were evaluated as described in the methods and the results are presented in Table 2 and Fig. 1A and B. In the heart tissues, normal histological findings were seen in the control group. On the other hand, there were histological changes in the DNR group. Qualitatively, DNR-induced cardiac damage was recognized by the presence of marked interstitial edema, myocardial fibrosis, perinuclear vacuolization, and degeneration of the myocardium. The lesions were significantly reduced in the group treated with telmisartan compared with those in the DNR group (Table 2 and Fig. 1A, B).

### Table 1. Changes in survival rate, histopathological, hemodynamic, and echocardiographic parameters after 6 weeks of treatment with telmisartan in DNR rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n=5)</th>
<th>DNR (n=12)</th>
<th>DNR+Telm (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survival rate (%)</td>
<td>100</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>540±8.9</td>
<td>379±9</td>
<td>380.5±8.4*</td>
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<tr>
<td>Heart Weight (g)</td>
<td>1.23±0.2</td>
<td>1.09±0.01*</td>
<td>0.95±0.03</td>
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<td>HW/BW ratio (g/kg)</td>
<td>2.3±0.2</td>
<td>2.9±0.06*</td>
<td>2.52±0.09</td>
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<tr>
<td><strong>Hemodynamic and Echocardiographic data</strong></td>
<td></td>
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<tr>
<td>CVP (mmHg)</td>
<td>-0.45±0.4</td>
<td>0.42±0.05</td>
<td>-0.26±0.36</td>
</tr>
<tr>
<td>MBP (mmHg)</td>
<td>95±7.6</td>
<td>85±5.6</td>
<td>85.93±5.56</td>
</tr>
<tr>
<td>LVP (mmHg)</td>
<td>124±3.6</td>
<td>111±7*</td>
<td>115.1±5.4</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>7.5±0.9</td>
<td>10.7±0.3*</td>
<td>8.7±0.4*</td>
</tr>
<tr>
<td>+dP/dt (mmHg/s)</td>
<td>6813±541</td>
<td>4800±545*</td>
<td>5950±398*</td>
</tr>
<tr>
<td>-dP/dt (mmHg/s)</td>
<td>7290±775</td>
<td>4135±365*</td>
<td>6079±739*</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>336±38</td>
<td>334±11</td>
<td>359.2±20.9</td>
</tr>
<tr>
<td>LVDd (mm)</td>
<td>6.6±0.5</td>
<td>7.5±0.3*</td>
<td>7±0.2</td>
</tr>
<tr>
<td>LVDs (mm)</td>
<td>4.4±0.3</td>
<td>5.3±0.2*</td>
<td>4.1±0.3*</td>
</tr>
<tr>
<td>FS (%)</td>
<td>42.8±1.7</td>
<td>29.1±1.3*</td>
<td>41±2.7*</td>
</tr>
<tr>
<td>EF (%)</td>
<td>78.9±1.8</td>
<td>59.6±1.4*</td>
<td>76.2±2.7*</td>
</tr>
</tbody>
</table>

Results are presented as the mean ± SEM. HW/BW, ratio of heart weight to body weight; CVP, central venous pressure; MBP, mean blood pressure; LVP, left ventricular pressure; LVEDP, left ventricular end-diastolic pressure; +dP/dt, rate of intra-ventricular pressure rise and decline; HR, heart rate; LVDd, left ventricular dimension in diastole; LVDs, left ventricular dimension in systole; FS, fractional shortening; EF, ejection fraction; group N, aged matched normal rats; group DNR, DNR rats treated with vehicle; group DNR+Telm, DNR rats treated with telmisartan (10 mg/kg/day).

*P < 0.05 vs. group control and #P < 0.05 vs. group DNR

### Table 2. Effect of telmisartan on histopathological changes in cardiac tissues after 6 weeks of treatment in DNR rats

<table>
<thead>
<tr>
<th>Histopathological finding</th>
<th>Control (n=5)</th>
<th>DNR (n=6)</th>
<th>DNR+Telm (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myocardial fibrosis</td>
<td>0±0.0</td>
<td>1.4±0.24*</td>
<td>0.6±0.24*</td>
</tr>
<tr>
<td>Perinuclear vacuolization</td>
<td>0±0.0</td>
<td>0.6±0.24*</td>
<td>0.4±0.24*</td>
</tr>
<tr>
<td>Myocardial degeneration</td>
<td>0±0.0</td>
<td>2.6±0.24*</td>
<td>1.2±0.2*</td>
</tr>
<tr>
<td>Interstitial edema</td>
<td>0±0.0</td>
<td>2.6±0.24*</td>
<td>1.4±0.24*</td>
</tr>
</tbody>
</table>

Results are presented as the mean ± SEM. group N, aged matched normal rats; group DNR, DNR rats treated with vehicle; group DNR+Telm, DNR rats treated with telmisartan (10 mg/kg/day). *P < 0.05 vs. group control and #P < 0.05 vs. group DNR.
Effect of telmisartan on myocardial level of pentosidine, and RAGE

Myocardial immunoreactivity as assessed by immunohistochemistry for pentosidine, and RAGE were increased in the group DNR compared with those of group Control, which were attenuated with telmisartan cotreatment (Fig. 2A and B).

Effect of telmisartan on myocardial protein expressions of CML and RAGE

Myocardial protein expression of CML and RAGE as assessed by western blotting was decreased in group DNR compared with that in group Control, and telmisartan treatment significantly attenuated the decrease in protein expressions of CML and RAGE (Fig. 3A and B).

Figure 1. A. Hematoxylin and eosin staining of the cross-sectional tissue slices of hearts depicting interstitial edema, vacuolization and degeneration of cardiac fibers (X200). B, Azan-Mallory staining for fibrosis of the cross-sectional tissue slices of hearts. Fibrosis is indicated by the blue area as opposed to the red myocardium (X200).

Figure 2. Immunohistochemical staining for pentosidine (A) and RAGE (B) in LV section (X400). Antibodies were detected by diaminobenzidine method that produces a brown color (arrow). Group CTL, age-matched normal rats; group DNR, DNR-treated rats administered with vehicle; group DNR+TLM, DNR-treated rats administered with telmisartan (10 mg/kg/day).
DISCUSSION

Although anthracycline-induced free radicals are believed to play a central role in its cardiotoxicity,\(^1\) the precise mechanism of myocardial impairment remains unclear. It is hoped that an enhanced understanding of the mechanisms underlying DNR-induced cardiomyopathy will enable the development of novel therapies that may prevent and treat the heart failure. In the present study, we confirmed—to our knowledge, for the first time—the contribution of AGE formation in DNR-induced cardiomyopathy in rats and that addition of telmisartan, a unique ARB with PPAR-\(\gamma\)-modulating activity during treatment with DNR reduced its cardiac damage in vivo.

In this study, DNR-induced cardiomyopathy was characterized by a deterioration of cardiac function as indicated by the deterioration in hemodynamic (LVP, LVDP, and dP/dt) and echocardiographic parameters (FS and EF) and confirmed by a severe histopathological features in the heart (Fig 1A and B and Table 1 and 2) which was in agreement with previous reports.\(^{11,13}\) Concomitantly, in the present study involvement of AGE formation in DNR-induced cardiomyopathy in rats and that addition of telmisartan, a unique ARB with PPAR-\(\gamma\) modulating activity during treatment with DNR reduced its cardiac damage in vivo.

In DOX-induced cardiomyopathy. The formation of pentosidine and CML is thought to be closely linked, not only to glycation, but also to oxidative stress.\(^{20}\) In addition, the final step of the Maillard reaction is driven by oxidative stress.\(^{21}\) Since the contribution of oxidative stress on anthracyclines-induced cardiotoxicity has been reported by some investigators (22,23), the results of the present study suggest that AGE formation is involved in the DNR-induced cardiomyopathy and the enhanced AGE formation by DNR in this present study might be associated with the enhanced oxidative and nitrosative stress to the cardiomyocyte.

In the present study, we found that telmisartan treatment attenuated the increased myocardial protein expression of CML and myocardial immunostaining of pentosidine in DNR-rats. Blockade of AGE formation or accumulation by renin-angiotensin system (RAS) inhibitors was reported both in vitro and in vivo.\(^{9,24}\) Olmesartan has been reported to inhibit the formation of pentosidine and CML by chelating transition metals and inhibits various oxidative steps in vitro.\(^9\) However, no study reported the crosstalk between RAS and the AGE-RAGE axis in anthracycline-induced cardiomyopathy. Telmisartan has been proven to inhibit intracellular oxidative stress, at least in part, in a receptor-independent manner, possibly owing to its lipophilic and anti-oxidant structure.\(^{27}\) In type I diabetic patients, telmisartan succeeded to improve endothelial function and decrease the nitrotyrosine plasma levels.\(^{28}\)
Thus, the mechanisms underlying the cardioprotective effects of telmisartan in our model may, in part, be due to the reduction in the formation of pentosidine and CML and oxidative stress.

Recent studies have shown that AGEs and their receptor (RAGE) axis is implicated in the pathogenesis of various devastating disorders such as diabetic, cardiovascular disease, and cancer growth and metastasis, but only a few reports outlining the contribution of AGE formation and RAGE to the adverse effects of medicinal drugs. Therefore, we further examined whether DNR could increased RAGE expression in the heart tissue. As shown in Figs. 2B, 4A, and B, RAGE protein expression assessed by Western blotting and immunohistochemical staining in the heart tissue was increased in DNR-rats which was inhibited by cotreatment with telmisartan. The present findings have extended previous observations that was inhibited by cotreatment with telmisartan. The staining in the heart tissue was increased in DNR-rats assessed by Western blotting and immunohistochemical shown in Figs. 2B, 4A, and B, RAGE protein expression increased RAGE expression in the heart tissue. As

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In conclusion, the formation of AGEs; pentosidine and CML might accelerates in DNR-induced cardiotoxicity and telmisartan could inhibits the progression of DNR-induced cardiomyopathy probably by alteration of AGEs formation through their receptor, RAGE.

Acknowledgments

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