E
lectrical and structural remodeling of the heart can develop in various pathological conditions, such as myocardial ischemia,1–3 cardiomyopathy,4,5 congestive heart failure,6,7 hypertension as well as myocarditis.8,9 In previous reports, we documented ventricular electrical and structural remodeling in an experimental autoimmune myocarditis (EAM) model in rats,8,9 and it was characterized by prolongation in the effective refractory period (ERP) and monophasic action potential duration (MAPD) and downregulated expressions of the Kv4.2, potassium channel interacting protein-2 (KChIP2) and sarcoplasmic reticulum Ca2+-ATPase 2a (SERCa2a). In this model, the overexpression of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) or interferon-γ (IFN-γ) induces myocardial damage and possibly causes ventricular remodeling.10,11 We have also reported that TNF-α stimulation induces cellular hypertrophy as well as suppression of the Ito current and Kv4.2 expression in cultured neonatal rat myocytes.12 Because TNF-α is a strong inducer of nitric oxide (NO) or reactive oxygen species (ROS), this inflammatory process may promote cardiac injury and electrical remodeling through a hyper-oxidative condition. Several studies have documented that antioxidant treatment using N-acetylcysteine (NAC), a thiol-containing radical scavenger and glutathione precursor, may attenuate the myocardial damage through in vitro and in vivo models of heart diseases,13–15 so that the anti-remodeling effect of antioxidant therapy might be expected in myocarditis. In the present study, we evaluated the expression of inflammatory cytokines and ROS generation through the development of cardiac remodeling in an EAM rat model, and also evaluated the effect of NAC as an antioxidative therapy for ventricular electrical and structural remodeling.

**Background:** Electrical and structural remodeling, characterized by prolonged action potential duration (APD), Kv4.2 downregulation and cellular infiltration were studied in rat experimental autoimmune myocarditis (EAM). Because the reactive oxygen species (ROS) has been speculated to play a role in the promotion of such remodeling, the effect of N-acetylcysteine (NAC) on the progression of ventricular remodeling was evaluated.

**Methods and Results:** Six-week-old Lewis rats were immunized with porcine cardiac myosin. On Days 10–11 after the immunization, NAC (0, 1, 10, or 100mg) was injected intraperitoneally to EAM and control rats. On Day 14, the electrophysiological parameters were evaluated and the expression levels of the mRNA were examined by quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR). The EAM rats exhibited a typical acute myocarditis with prolonged APD and reduced Kv4.2 expression as previously reported. The myocarditis and electrical changes were significantly suppressed by NAC-treatment in a dose-dependent manner (P<0.05). In rats with 100mg NAC, the myocarditis was almost totally negated although the mortality increased. In rats with 1mg NAC, the suppression of myocarditis was not obvious, but APD prolongation and Kv4.2 reduction was attenuated (P<0.05).

**Conclusions:** The NAC treatment suppressed ventricular remodeling in the EAM rats. This may indicate the role of oxidative stress in causing remodeling and myocarditis itself in the acute phase of myocarditis. (Circ J 2011; 75: 662–671)

**Key Words:** Myocardial remodeling; Myocarditis; N-acetylcysteine; Radical scavenger; Reactive oxygen species

N-Acetylcysteine Suppresses the Progression of Ventricular Remodeling in Acute Myocarditis
– Studies in an Experimental Autoimmune Myocarditis (EAM) Model –

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**Methods**

**Immunization and NAC Treatment**

Six-week-old Lewis rats were immunized with purified porcine cardiac myosin mixed with a Freund’s complete adjuvant in each rear footpad on Day 1 as previously described.8,9 The control rats received injections of 0.25 ml of saline in the same manner. The rats with immunization (ie, EAM rats) were divided into 5 groups. The first group was sacrificed on Day 10 to evaluate the macroscopic and histological findings in that phase. The remaining 4 groups received intraperitoneal injections of a saline solution that contained doses of NAC of 0, 1, 10 or 100 mg · body−1 · day−1 on Days 10 and 11. This NAC injection phase was selected because myocarditis is not obvious yet (ie, the antigen priming phase). The control rats were divided into 4 groups and received NAC injections similar to the latter 4 groups of EAM rats. All studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and Ethics Committee of the Kitasato University School of Medicine.

**Hemodynamic and Electrophysiological Parameters**

The first EAM group was sacrificed on Day 10 after the initial immunization and the whole hearts were excised for histological analysis. In the remaining rats, the trans-thoracic echocardiogram was recorded with a 7.5-MHz imaging transducer (Pro-Sound SSD-4000, ALOKA, Tokyo, Japan) without anesthesia on Day 14. M-mode recording was performed at the papillary muscle level to evaluate the left ventricular ejection fraction (LVEF) and fraction shortening (%FS). After the echocardiographic evaluation, the heart was exposed by a median sternotomy under interperitoneal anesthesia with pentobarbital. The ventricular electrograms were recorded using a pair of platinum needle electrodes (0.1 mm) as previously described.8,9 The analogue signals were converted into digital signals at a sampling frequency of 1,000 Hz (Power Lab 8sp, Bio Research Co Ltd, Tokyo, Japan) and stored on a computer hard disk.18 The band pass filter was set at 50–300 Hz for standard cardiac electrogram recordings and at an open-300 Hz for recording the monophasic action potential (MAP). The heart rate was evaluated during stable anesthesia. To evaluate the ventricular ERP, a 2-ms step shortening of the coupling interval of the extrastimulus was employed in 2 basic cycle lengths of 150 and 120 ms. The duration of the MAP (MAPD) was determined as the interval between the onset of the MAP trace and 20% (MAPD20%) and 90% repolarization times (MAPD90%).8,9 For the hemodynamic parameters, the left ventricular systolic pressure (LVSP) and left ventricular end-diastolic pressure (LVEDP) were monitored by a needle tip micromanometer (SPR477, Millar, USA) as previously described.8,9 The heart tissue evaluated in this invasive protocol was not used for samples for the other analyses.

**Heart Weight and Histology**

The first EAM group was sacrificed on Day 10. The remaining groups were sacrificed on Day 14 after the initial immunization under anesthesia and the whole heart was excised. The degree of myocarditis was graded from 0 to 4 in accordance with the macroscopic scoring system as described previously.19 The weight of the whole heart was measured to calculate the ratio of heart and body weight (Hw/Bw). The heart was transversely sliced, fixed in 10% formalin solution and stained with H&E. The area of the entire heart and the affected region by myocarditis (ie, regions showing infiltration of inflammatory cells and myocardial necrosis) were measured to calculate the microscopic score as previously described.19 To evaluate the oxidative stress in the myocardial tissue, N-[Hexanoyl]-lysin (HEL) staining was performed. The whole heart of 4 rats in each group were fixed in Bouin’s

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**Table 1. Heart Weight and Histopathology**

<table>
<thead>
<tr>
<th></th>
<th>Control (Day 14)</th>
<th>EAM (Day 14)</th>
<th>EAM (Day 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 mg NAC (n=7)</td>
<td>1 mg NAC (n=7)</td>
<td>10 mg NAC (n=7)</td>
</tr>
<tr>
<td>Hw/Bw (g/kg)</td>
<td>3.9±0.4</td>
<td>11.0±0.52</td>
<td>4.23±0.41</td>
</tr>
<tr>
<td>Microscopic grade</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>Grade 0 (n)</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Grade 1 (n)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Grade 2 (n)</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Grade 3 (n)</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Grade 4 (n)</td>
<td>0</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Microscopic grade</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>HEL staining</td>
<td>0.2±0.3</td>
<td>0.3±0.4</td>
<td>0.3±0.4</td>
</tr>
<tr>
<td>HO-1 mRNA expression</td>
<td>7.3±1.7</td>
<td>7.8±2.5</td>
<td>6.6±3.0</td>
</tr>
<tr>
<td>HO-1/TNT copy ratio (×10⁶)</td>
<td>2.3±0.5</td>
<td>2.3±0.6</td>
<td>2.0±0.7</td>
</tr>
</tbody>
</table>

*P<0.05 vs. control (Day 14); †P<0.05 vs. EAM + 0 mg NAC (Day 14).
solution (water saturated with picric acid; 40% formalin; glacial acetic acid at a ratio of 75:15:5) at 4°C. Paraffin-embedded tissues were cut and stained with an anti-HEL monoclonal antibody (NIKKEN SEIL Co, Ltd, Tokyo, Japan). For quantitative evaluation of the expression of oxidative stress, the HEL-stained area ratio was measured with the NIH imaging system. The mean value was calculated from the data of 50 randomly selected microscopic areas. To verify ROS expression in the ventricle, the mRNA levels of hemoglobinase-1 (HO-1) were evaluated using the following quantitative real-time RT-PCR method. 21

**Table 2. Hemodynamic and Electrophysiological Parameters**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control (Day 14)</th>
<th>EAM (Day 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (beats/min)</td>
<td>380±42 (n=7)</td>
<td>100 mg NAC (n=7)</td>
</tr>
<tr>
<td>LVSP (mmHg)</td>
<td>124±26 (n=7)</td>
<td>100 mg NAC (n=7)</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>2.0±1.0 (n=7)</td>
<td>100 mg NAC (n=7)</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>68±4 (n=7)</td>
<td>100 mg NAC (n=7)</td>
</tr>
<tr>
<td>LV %FS</td>
<td>44±3 (n=7)</td>
<td>100 mg NAC (n=7)</td>
</tr>
<tr>
<td>ERP (BCL=150 ms, ms)</td>
<td>68±15 (n=7)</td>
<td>100 mg NAC (n=7)</td>
</tr>
<tr>
<td>ERP (BCL=120 ms, ms)</td>
<td>64±12 (n=7)</td>
<td>100 mg NAC (n=7)</td>
</tr>
<tr>
<td>MAPD 664 (BCL=150 ms, ms)</td>
<td>14±4 (n=7)</td>
<td>100 mg NAC (n=7)</td>
</tr>
<tr>
<td>MAPD 664 (BCL=120 ms, ms)</td>
<td>15±4 (n=7)</td>
<td>100 mg NAC (n=7)</td>
</tr>
<tr>
<td>MAPD 664 (BCL=150 ms, ms)</td>
<td>64±8 (n=7)</td>
<td>100 mg NAC (n=7)</td>
</tr>
<tr>
<td>MAPD 664 (BCL=120 ms, ms)</td>
<td>62±6 (n=7)</td>
<td>100 mg NAC (n=7)</td>
</tr>
</tbody>
</table>

*P<0.05 vs. control; †P<0.05 vs. EAM + 0 mg NAC. LVSP, left ventricular systolic pressure; BCL, basic cycle length.

**Table 3. Absolute Copy Numbers of mRNA in Rat Hearts With N-Acetylcysteine Treatment**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control (Day 14)</th>
<th>EAM (Day 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kv4.2 x10^6</td>
<td>16.6±1.3 (n=10)</td>
<td>10 mg NAC (n=10)</td>
</tr>
<tr>
<td>Kv1.5 x10^6</td>
<td>8.2±0.9 (n=10)</td>
<td>10 mg NAC (n=10)</td>
</tr>
<tr>
<td>Kv4.3 x10^6</td>
<td>14.6±1.6 (n=10)</td>
<td>10 mg NAC (n=10)</td>
</tr>
<tr>
<td>Kv1.4 x10^6</td>
<td>20.4±1.6 (n=10)</td>
<td>10 mg NAC (n=10)</td>
</tr>
<tr>
<td>Erg x10^6</td>
<td>36.4±4.2 (n=10)</td>
<td>10 mg NAC (n=10)</td>
</tr>
<tr>
<td>TNT x10^6</td>
<td>32.4±3.3 (n=10)</td>
<td>10 mg NAC (n=10)</td>
</tr>
<tr>
<td>BNP x10^6</td>
<td>25.5±3.2 (n=10)</td>
<td>10 mg NAC (n=10)</td>
</tr>
<tr>
<td>SERCAa x10^6</td>
<td>8.6±1.2 (n=10)</td>
<td>10 mg NAC (n=10)</td>
</tr>
<tr>
<td>RyR x10^6</td>
<td>15.6±2.2 (n=10)</td>
<td>10 mg NAC (n=10)</td>
</tr>
<tr>
<td>NCX x10^6</td>
<td>18.2±2.2 (n=10)</td>
<td>10 mg NAC (n=10)</td>
</tr>
<tr>
<td>L-Ca^2+ x10^6</td>
<td>22.2±2.6 (n=10)</td>
<td>10 mg NAC (n=10)</td>
</tr>
<tr>
<td>Frenquin x10^6</td>
<td>15.4±1.6 (n=10)</td>
<td>10 mg NAC (n=10)</td>
</tr>
<tr>
<td>KChIP2 x10^6</td>
<td>18.6±2.1 (n=10)</td>
<td>10 mg NAC (n=10)</td>
</tr>
<tr>
<td>TNF-α x10^6</td>
<td>2.9±1.5 (n=10)</td>
<td>10 mg NAC (n=10)</td>
</tr>
<tr>
<td>IL-10 x10^6</td>
<td>16.8±1.6 (n=10)</td>
<td>10 mg NAC (n=10)</td>
</tr>
<tr>
<td>IFN-γ x10^6</td>
<td>22.4±6.2 (n=10)</td>
<td>10 mg NAC (n=10)</td>
</tr>
</tbody>
</table>

*P<0.05 vs. control; †P<0.05 vs. EAM + 0 mg NAC.

Quantitative Real-Time RT-PCR

The ventricular tissue was sampled from the sacrificed rats mainly on Day 14 after the initial immunization and on Day 10 for the evaluation of ROS expression on that phase. Total RNA was purified from the left ventricular free wall using a total RNA isolation kit (SV Total RNA Isolation System, Promega, USA). cDNA was synthesized from 3 μg of the total RNA with reverse transcriptase (Invitrogen, CA, USA) in a final volume of 20 μl. The mRNA levels of the voltage dependent K+ channels (Kv1.4, 4.2, 4.3, 1.5 and erg), Ito-regulating molecules (K+ channel-interacting protein-2; KChIP2 and neuronal calcium channel protein-1; frequnin), cardiac ion transporters (sarco/plemic reticulum Ca2+ ATPase 2a; SERCA2a, ryanodine receptor; RyR and Na+/Ca2+ exchanger; NCX, L-type Ca2+ channel), inflammatory cytokines (TNF-α, IFN-γ and IL-10), BNP and HO-1 were evaluated by quantitative real-time RT-PCR. To estimate the loss of myocardium, the level of troponin T (TNT) mRNA was also measured. 8 The real-time RT-PCR was performed with a QuantiTectTM SYBR Green PCR Master Mix (Qiagen, CA, USA) using a CFD 3240 Chromo4TM Detection System (Bio-Rad Lab, Inc, USA). To make the standard curve, the standard plasmid for each molecule was constructed as previously described. 21 Serially diluted standard plasmids were analyzed at the same time and absolute copy numbers were calculated. 9
Numbers of Rats in Each Study Protocol

In total, 85 rats were immunized and autoimmune myocarditis was induced in all rats. Of the 85 rats, 7 were used for Day 10 sacrifice and 23, 17, 17 and 21 for the 0 mg, 1 mg, 10 mg and 100 mg NAC treatments, respectively. Because 1 of 23 rats with 0 mg NAC treatment and 4 of 21 rats with 100 mg NAC treatment died during observation, the remaining rats were used for the further study. As a result, the number of rats for heart tissue sampling was 7 for Day 10 sacrifice and 15, 10, 10 and 10 for the 0 mg, 1 mg, 10 mg and 100 mg NAC treatments, respectively. The number of rats for the invasive study was 7 for each of the latter 4 groups. As the control, 59 rats given saline injections were used. Of the 59 rats, 14, 14, 14 and 17 were used for the 0 mg, 1 mg, 10 mg and 100 mg NAC treatments, respectively. Because 3 of 17 rats with the 100 mg NAC treatment died during observation, the remaining rats were used for the further study. As a result, the number of rats for the heart tissue sampling and invasive study was 7 for each group (Tables 1–3).

Statistical Analysis

All quantitative data are described as the mean±SEM. Differences among groups were tested by a one-way ANOVA and P<0.05 was considered to be statistically significant.

Results

Mortality of the Rats During the Protocol

As described in the method section, 1/23 (4.3%) rats with an EAM+0mg NAC treatment died suddenly on Day 13 probably because of heart failure from acute myocarditis. In contrast, 4/21 (19.0%) rats with an EAM+100mg NAC treatment and 3/17 (17.6%) control rats with 100 mg NAC treatment died suddenly after the second NAC injection on Day 11. These mortalities in the groups with 100 mg NAC injections were significantly higher than in the other groups (P=0.027). In the autopsy of these rats, no macroscopic or microscopic abnormalities were observed in the major organs except slight ascites. None of the EAM rats for Day 10 sacrifice died during the protocol.

Histopathology and ROS Expression

Figure 1 exhibits the representative examples of the histopathological findings of rat hearts. Only the left column exhibits the EAM data of rats on Day 10, but all of the remaining panels show the data on Day 14. In comparison with the control rat with 0 mg of NAC (B, G, L, R), the heart from the EAM+0 mg NAC rat exhibits a marked enlargement and spotty changes to the surface color as the result of acute myocarditis in the macroscopic findings (C), infiltration of inflammatory cells in the HE staining (H&M) and brown staining as a result of a hyper-oxidative state in the HEL staining (S). In the hearts from the EAM+10 mg NAC (D, I, O, T) and 100 mg NAC (E, J, P, U) rats, these findings of acute myocarditis were suppressed in a dose-dependent manner.
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control + 0 mg NAC rats (Figure 1B) and spotty changes in the surface color could be observed as the result of infiltration of inflammatory cells and myocardial necrosis. In the rat hearts with EAM + 10 mg NAC (Figure 1D) and 100 mg NAC (Figure 1E) treatments, these findings of acute myocarditis were suppressed in a dose-dependent manner. In the H&E staining, the whole heart still looked normal on Day 10 (Figure 1F), but microscopic findings exhibit a small amount of intercellular edema and infiltration of mono-nuclear cells (Figure 1K) indicating a very early phase of inflammation. On Day 14, marked infiltration of inflammatory cells was observed in the ventricular myocardium in the rat hearts with an EAM + 0 mg NAC treatment (Figures 1H, M). In the rat hearts with EAM + 10 mg NAC (Figures 1I, O) and 100 mg NAC (Figures 1J, P) treatments, inflammatory cellular infiltration was suppressed in a dose-dependent manner. In the HEL staining, weak but obvious brown staining could be observed around the epicardial surface of the myocardium on Day 10 (Figure 1Q). On Day 14, the brown staining became stronger than at Day 10, and could be observed in the myocardium in the EAM + 0 mg NAC (Figure 1S) and 10 mg NAC (Figure 1T) rats indicating a hyper-oxidative state in the cytoplasm and cell membrane.

Table 1 summarizes the heart and body weight ratio, histopathological findings and ROS expressions. The right column shows the data of EAM rats on Day 10, and all of the remaining data exhibit the data on Day 14. EAM rats exhibited an increase in the Hw/Bw compared with the controls on Day 14, but it was not obvious on Day 10. This increase in Hw/Bw was suppressed by an NAC injection of 100 mg. The EAM + 0 mg NAC rats on Day 10 did not show obvious macroscopic myocarditis, but a small amount of inflammatory cellular infiltration was observed as shown in Figure 1. In contrast, the EAM + 0 mg NAC rats on Day 14 exhibited typical findings of acute myocarditis characterized by heart enlargement and inflammatory cellular infiltration as shown in Figure 1, and the macroscopic and microscopic grades of myocarditis were significantly higher than in the control. These findings of acute myocarditis were suppressed by an NAC injection in a dose-dependent manner, but suppression became significant with a 100-mg injection of NAC in the macroscopic findings, and with a 10-mg injection of NAC in the microscopic findings. In the HEL staining, brown staining as a result of a hyper-oxidative state was observed in the EAM rats on Day 14, and was suppressed by NAC treatment in a dose-dependent manner. This staining could already be observed on Day 10, although the degree was smaller than on Day 14. The quantitative evaluation of HO-1 mRNA expression by the real-time RT-PCR also exhibited increased ROS expression on Days 10 and 14 in EAM rats, but it was suppressed by NAC injection in a dose-dependent manner (Table 1).

**Figure 2.** Representative traces of ventricular monophasic action potentials and effective refractory period (ERP) data. This figure exhibits the representative examples of traces of monophasic action potentials (MAPs) recorded from the left ventricle and ERP data from each group. The EAM + 0 mg NAC rats exhibited a prolongation of the MAP duration (MAPD) and ERP and those prolongations were suppressed by an NAC injection in a dose-dependent manner. See the text for the details. BCL, basic cycle length.
Hemodynamic Parameters

Table 2 summarizes the hemodynamic and electrophysiological parameters evaluated in 7 rats in each group on Day 14. There were no significant differences in the heart rate and LVSP among the groups. The LVEDP was increased, and the LVEF and LV-%FS were decreased in the EAM groups compared with the control. An NAC injection suppressed these changes in a dose-dependent manner, but the suppression became significant with 10 mg or 100 mg injections of NAC.

Electrophysiological Parameters

Figure 2 exhibits representative examples of traces of monophasic action potentials (MAPs) recorded from the left ventricle and ERP data of each group on Day 14. As we previously reported, the EAM + 0 mg NAC rats exhibited a prolongation of the MAP duration (MAPD) and ERP. As shown in the left panel, this prolongation was suppressed by an NAC injection in a dose-dependent manner. In the ERP data, the suppressive effect of NAC on the ERP prolongation became significant with 10-mg or 100-mg injections. Figure 3 exhibits the MAPD data of each group. Similar to the ERP data, the MAPD was prolonged in the EAM groups compared with the control, and this prolongation was suppressed by an NAC injection in a dose-dependent manner. But at that time, the suppressive effect of NAC on the MAPD prolongation was significant even with a 1-mg injection of NAC, which was the smallest dose of NAC, with at least a basic cycle length (BCL) of 150 ms.

Quantitative Real-Time RT-PCR

The absolute copy numbers of the 16 species of the mRNA in the samples are summarized in Table 3. As previously described, we used troponin-T (TNT) mRNA as the internal control because it is highly specific to myocytes, and a usual internal control, such as cyclophilin or G6PDH, may overestimate the downregulation of myocyte specific genes due to the influence of inflammatory cellular infiltration. Out of the molecules evaluated in this study, Kv4.2, Kv1.5, Kv4.3, SERCA2a and KChIP2 were significantly downregulated in the EAM + 0 mg NAC rats even after the correction by the TNT mRNA. The results of the corrections of Kv4.2, Kv1.5, SERCA2a and KChIP2 are shown in Figure 4. These downregulations were suppressed by the NAC injection in a dose-dependent manner. Interestingly, the suppressive effect of NAC on the downregulation of Kv4.2 and KChIP2 was significant even with 1 mg of NAC (ie, the smallest dose in this study), although the suppressive effect became significant only with a 100-mg dose of NAC for Kv1.5 and SERCA2a.

In contrast, the mRNA expressions of the BNP and inflammatory cytokines (ie, TNF-α, IFN-γ and IL-10) were upregulated in the EAM + 0 mg NAC rats compared with the control + 0 mg NAC rats. The results are shown in Figure 5. The upregulation of the mRNA of the BNP and inflammatory cytokines were suppressed by the NAC injection in a dose-dependent manner, but this suppression was significant with 10 mg or 100 mg of NAC, but not with 1 mg of NAC.

Figure 3. Change in the duration of the monophasic action potentials. This figure exhibits the monophasic action potential duration (MAPD) data from each group. Similar to the effective refractory period (ERP) data, the MAPD was prolonged in the experimental autoimmune myocarditis (EAM) groups compared with the control, and this prolongation was suppressed by an NAC injection in a dose-dependent manner. But at that time, the suppressive effect of NAC on the MAPD prolongation was significant even with a 1-mg injection of NAC, which was the smallest dose of NAC, with at least a basic cycle length (BCL) of 150 ms.
Discussion

This study evaluating the effects of NAC on ventricular electrical and structural remodeling in EAM rats exhibited several important findings. First, the EAM rats exhibited typical acute myocarditis on Day 14 after the initial immunization and electrical and structural remodeling characterized by the prolongation of the ERP and MAPD as well as tissue edema with inflammatory cellular infiltration. As the basis of the electrical remodeling, the downregulation of Kv4.2, Kv1.5, SERCA2a and KChIP2 mRNA were documented as shown in our previous report.9

Second, intraperitoneal NAC injections on Days 10 and 11 after the initial immunization suppressed the myocarditis and electrical remodeling in a dose-dependent manner. To the best of our knowledge, this is the first report documenting a drastic suppression of myocarditis and its ventricular remodeling by the transient use of antioxidants.

Third, especially in rats with an EAM + 1 or 10 mg NAC injection, which were the smaller doses in this study, the myocarditis was not obviously suppressed but the electrical remodeling was partly suppressed. These findings indicate that hyper-oxidative stress plays a role in promoting electrical and structural remodeling in EAM.

Role of Oxidative Stress in the Development of Myocarditis

The effect of NAC on cardiac diseases is controversial.24–27 However, at least in the present study, the use of NAC suppressed the progression of myocarditis in a rat EAM model. Theoretically, the primary antioxidant effects of NAC reduce the ROS production and inhibit the activation of nuclear factor-κ B (NFκB) by replenishing glutathione contents. In addition, because glutathione is an inhibitor of the TNF-α/TNF-receptor1/shphingomyelinase signaling cycle, it results in the suppression of the cytokine–chemokine network in cardiac tissue.14,28 These effects of NAC may explain its suppressive effect on myocarditis especially with a middle or higher dose, but an additional theory would be necessary because only a 2-day administration of NAC successfully suppressed the development of myocarditis in the present study. Nimata et al reported that the use of a radical scavenger, edaravone, could suppress the development of myocarditis in the same EAM rat.29 The results were well compatible with the results in the present study, but the phase of the treatment was different. We evaluated the histological findings and expression of oxidative stress in EAM on Day 10 after the immunization. The result was that the macroscopic myocarditis was not obvious in this phase, but a really small amount of intercellular edema and infiltration of mono-nuclear cells were observed in histology, and HEL staining indicates a weak ROS expression around the epicardial surface of the myocardium. These results indicate that Days 10–11 would be the pre- or early-myocarditis phase, which would appear with hyper-oxidative stress. The source of ROS is unclear, but infiltrating mono-nuclear cells, such as monocytes, might be speculated as the source by referring histological findings on Day 10.
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Phase may be the effector phase of the auto-immune process when the interaction between effector T cells and antigen presenting cells such as dendritic cells has been activated, which is a major trigger for the development of myocardial injury. Matsue et al demonstrated the generation of ROS in dendritic cells and T cells during antigen presentation and they also documented that the antioxidant suppressed the interaction between the dendritic cells and T cells in vitro in an animal model of allergic contact dermatitis. It can be speculated that the use of antioxidants during this specific phase of EAM would suppress several autoimmune processes, and may then result in the suppression of the development of myocarditis. We cannot conclude anything about the effect of antioxidants on the induction phase (ie, Days 1–9 after the immunization) through the results of the present study, but the early effector phase (ie, Days 10–11) may have a specific role in the promotion of further myocarditis by referring the report of Shioji et al, which documented the importance of Days 10–14 for suppression of myocarditis using immunoglobulin.

However, it should be considered that antioxidants do not always work in a useful way. In the present study, the 100 mg injection of NAC, which was the highest dose, caused a considerable number of sudden deaths both in the control and EAM rats. This may indicate that over-suppression of oxidative stress may suppress the basic cellular function, and may lead to a toxic function of NAC. Therefore, the degree of antioxidants should be an important issue in considering antioxidants as the clinical therapy.

Role of Oxidative Stress in Electrical Remodeling
In the present study, we documented that an NAC injection suppressed ventricular electrical remodeling, which was characterized by the prolongation of ERP and MAPD as well as the downregulation of Kv4.2, Kv1.5, Kv4.3, SERCA2a and KChIP2. The suppression of electrical remodeling by the NAC might be understood as being the result of the suppression of the myocarditis. However, by considering the results in the EAM + 1 mg NAC rats (ie, the smallest dose), an additional theory is necessary. The result was that the prolongation of MAPD20 and MAPD90 at the basic cycle length of 150 ms and the downregulation of Kv4.2 and KChIP2 were significantly suppressed even by a 1-mg injection of NAC, but the suppression of the myocarditis itself was not obvious at that dose of NAC. Therefore, antioxidants may directly suppress the electrical remodeling and not only through the suppression of the infiltration of inflammatory cells.

It has been reported that stimulation of TNF-α, angiotensin II or stretch can increase protein catabolism and cardiac protein synthesis, and it leads to cellular hypertrophy, apoptosis and inflammation by increased ROS activity. We previously reported that TNF-α stimulation downregulates the voltage gated outward K+ current in cultured neonatal rat cardiomyocytes, and Higuchi et al reported that NAC inhibited TNF-α-induced cellular hypertrophy and increased protein synthesis. Considering the results of these reports, ROS activation can be considered to play an important role in promoting ventricular remodeling in the inflammatory condition.

We cannot conclude anything about the antioxidants other

![Figure 5. Changes in the mRNA expression of the BNP and inflammatory cytokines. This figure exhibits changes in the mRNA expression of the BNP and inflammatory cytokines. They were upregulated in the EAM + 0 mg NAC rats compared with the + 0 mg NAC control. The upregulation of the mRNA of the BNP and inflammatory cytokines was suppressed by an NAC injection in a dose-dependent manner, but that suppression only became significant with 10 mg or 100 mg of NAC, but not with 1 mg of NAC. See the text for the details.](image-url)
than NAC through the results of the present study, but other clinical antioxidants, such as edaravone or nicaraven, might be effective for preventing progression of myocardial remodeling in diseased models.21-34

Study Limitations
First, because the electrophysiological and hemodynamic parameters were evaluated under an open-chest anesthetized state, each parameter could have been influenced by the anesthesia. Second, because this study used an in vivo model, the importance of the specific signals for initiating ventricular remodeling, such as inflammation and ROS activity, could not be separated. This point could be addressed in future experiments by using cultured myocytes.

Third, the level of ROS expression was verified only through a single methodology using HEL staining. However, the quantitative evaluation was performed by a computer image analyzing system.

Conclusions
Ventricular electrical and structural remodeling was suppressed by an NAC injection at its effecter phase in the autoimmune process in EAM rats. Activation of ROS was considered to play an important role in the development of ventricular remodeling and myocarditis.

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Disclosure
There was no financial support from specific companies for this study or any conflict of interest, and no specific unaproved usage of any compound or product occurred.

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