ORIGINAL ARTICLE

AMIODARONE ATTENUATES THE UPREGULATED MATRIX METALLOPROTEINASE-2 ACTIVITY IN A RAT MYOCARDIAL INFARCTION MODEL

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Abstract Structural remodeling occurs in diverse heart diseases and affects their clinical courses. We reported that amiodarone suppresses both electrical and structural remodeling in a canine persistent atrial fibrillation model. As a mechanism for amiodarone's effect on structural remodeling, we suggested its inhibitory effect on matrix metalloproteinase（MMP）activity. To elucidate it, we investigated the effect of amiodarone on MMP activity in a rat myocardial infarction model created by left coronary artery（LCA）ligation. Adult Sprague-Dawley rats were divided into sham-operated（Sham）, sham-operated with amiodarone（Sham+AMD）, LCA-ligated without amiodarone（MI）and LCA-ligated with amiodarone rats（MI+AMD）. Amiodarone（20 mg/kg/day）was administered for 2 weeks before and for 4 weeks after operation. The hearts were excised at 4 weeks after operation. MMP-2 activity was measured by gelatin zymography. At 4 weeks after surgery, left ventricular fractional shortening was decreased in MI but not in MI+AMD rats. There was no difference in the infarct size between MI and MI+AMD rats（P=NS）. As compared with Sham, MMP-2 activity was increased in MI（$P<0.01$）, but not in MI+AMD（P=NS versus Sham; $P<0.05$ versus MI）. MMP-2 activity was not increased in Sham+AMD（P=NS）. Thus, amiodarone exerts an inhibitory effect on MMP activity. This may be related to the improvement left ventricular function in MI rats.

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Key words: structural remodeling; amiodarone; matrix metalloproteinases; myocardial infarction.

原 著

アミオダロンのマトリックスメタロプロテイナーゼ-2活性に対する作用 ：ラット心筋梗塞モデルでの検討

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抄録 構造的リモデリングの進展は心房細動や心筋梗塞をさらに悪化させる．以前我々は犬心房細動モデルでアミオダロン（AMD）が構造的リモデリングを抑制することを示した．そのリモデリング抑制機序に AMD のマトリックスメタロプロテイナーゼ（MMP）抑制作用が関与するかを，ラット心筋梗塞モデルで検証した．Sham 群，AMD を投与し sham を作成した Sham+AMD 群，プラセボを投与し冠動脈結紮した MI 群，および AMD を投与し冠動脈結紮した MI+AMD 群に分類し，術後4週目にザイモグラフィーでの MMP-2 活性を比較した．投薬は術前2週から術後4週まで継続した．術後4週目の左室内径短縮率は MI 群で低下したが，MI+AMD 群では低下しなかった．Sham 群に比し，MI 群の MMP-2 活性は増強していた（$P<0.01$）が，MI+AMD 群では有意差を認めなかった．心筋梗塞モデルで AMD は MMP-2 活性を抑制することが示され，心筋梗塞による心機能抑制の改善に関係することが示唆された．

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キーワード：構造的リモデリング；アミオダロン；マトリックスメタロプロテイナーゼ；心筋梗塞.

Introduction

Amiodarone was introduced as an antianginal drug in 1967 and as an antiarrhythmic drug in 1970.[1, 2] Several recent studies have demonstrated that amiodarone improves the clinical status and left ventricular function in patients with heart failure.[3, 4] In the European Myocardial Infarct Amiodarone Trial (EMIAT), although there was no improving effect on all-cause mortality and cardiac mortality, amiodarone showed a significant, 35% risk reduction in the presumed arrhythmic deaths.[5] In the meta-analysis of clinical trials, amiodarone was shown to decrease a total mortality rate by 13% in patients with myocardial infarction and/or with congestive heart failure.[6] Amiodarone exerts a potentially effective antiarrhythmic effect, but its efficacy in clinical cases especially in those with depressed cardiac function cannot be explained solely by the antiarrhythmic effect. Amiodarone has been shown to exert pleiotropic effects other than the antiarrhythmic effect,[7–17] and these may be related to its beneficial effect in clinical cases. However, the precise mechanism for the beneficial effect was not clarified yet.

Myocardial infarction often results in left ventricular (LV) dilatation and pump dysfunction, being associated with myocyte hypertrophy and interstitial fibrosis in the non-infarct zone. These chamber dilation and interstitial fibrosis, that is, structural remodeling, occurs in diverse heart diseases and affect their clinical courses.[18] Matrix metalloproteinases (MMPs) have been shown to contribute to the development of structural remodeling in the interstitial tissues.[19, 20] We reported that the circulating level of gelatinolysis activity predicts the development of ventricular remodeling in patients with acute myocardial infarction.[21] We also reported that amiodarone suppresses both electrical and structural remodeling in a canine persistent atrial fibrillation model.[22] As a mechanism for amiodarone's effect on structural remodeling, we suggested its inhibitory effect on MMP activity. In the present study, we tested the hypothesis that amiodarone attenuates the enhanced MMP activity occurring after myocardial infarction. For this purpose, we created a rat myocardial infarction model by ligation of the left coronary artery (LCA) and compared cardiac function and MMP activity between the animals with and without amiodarone treatment.

Methods

Experimental design

All studies were performed in accordance with Guidelines for Animal Experimentation of Hirosaki University. Twenty seven Sprague-Dawley rats (9 to 11 weeks old and 364 ± 11 g in body weight) were divided into sham-operated (Sham, n=9), sham-operated with amiodarone treatment (Sham+AMD, n=4), LCA-ligated without amiodarone (MI, n=7) and LCA-ligated with amiodarone rats (MI+AMD, n=7). Amiodarone (20 mg/kg per day) prepared fresh daily as a micro suspension in tepid water or placebo was administered orally once a day for the 2-week period before operation and for the additional 4-week period after operation.

Myocardial infarction was created by ligating the LCA as described previously.[23] Briefly, rats were anesthetized by intraperitoneal injection with pentobarbital (30 mg/kg), intubated with 16-gauge polyethylene catheter, and ventilated with room air. With the use of sterile techniques, the chest was opened along the left sternal border, and the fourth rib was cut proximal to the sternum. Using a 6-0 suture, the LCA was ligated at the site between the pulmonary artery cone and the left atrial appendage. This procedure generated a similar size of myocardial infarction throughout the experiments. Myocardial ischemia caused by ligation of the LCA was confirmed visually by the change in the color of the myocardium. In sham-operated group, the same

procedure was performed except for the ligation of LCA. Finally, the chest wall was closed and the animals were allowed to recover.

At 4 weeks after surgery, rats were euthanized, the chest was opened, and the heart and lungs were harvested and weighed. Nine Sham rats, 4 Sham+AMD rats, 7 MI rats, and 7 MI+AMD rats were used for the subsequent biochemical studies. The ventricular myocardial tissue was carefully dissected into two parts, one consisting of the infarct LV and the other of non-infarct LV. The differentiation of the infarct and non-infarct LV was made macroscopically based on the finding that the infarct myocardium changed to the white-color scar lesion. In the subsequent assay, the comparison was made between non-infarct LV obtained from MI rats and intact LV from Sham rats.

Echocardiographic and hemodynamic measurements

Echocardiographic and physiological measurements were performed at baseline, just before operation, and 4 weeks after operation. Under light anesthesia with an excessive inhalation of diethylether and spontaneous respiration, transthoracic echocardiography was recorded with the use of Aspen Imagegate (Acuson, California, USA) equipped with a 5- to 10-MHz linear probe. The LV was imaged in parasternal long-axis view and measured digitally in M-mode. LV end-diastolic diameter (LVDd) was defined as the largest LV diameter, systolic diameter (LVDs) was defined as the smallest LV diameter, and the LV fractional shortening (LVFS) was calculated as [(LVDd-LVDs)/LVDd] x 100. The data obtained from at least three cardiac cycles were averaged and used for analysis. Heart rate (in beats/min) and blood pressure (in mmHg) were determined in the conscious state using a computerized tail-cuff manometer (Softron BP-98A; Softron Co., Tokyo, Japan).

SDS-PAGE zymography of the myocardial tissues

The non-infarct LV myocardial samples were homogenized and centrifuged and the supernatant was corrected as described previously.[19, 24] MMP activity was measured by gelatin zymography.[25] Briefly, the supernatant samples were separated by dilution into zymography sample buffer. The samples and MMP-2 standard (1.25x 10^{-3} unit/lane, Wako chemical, Japan) were electrophoresed in a 10% gelatin gel, and incubated in renaturing buffer (2.5% Triton X-100). The gel was incubated with development buffer (50 mM Tris, pH 7.5, 200 mM NaCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.02 % Briji-35) at 37 ℃ for 18 hours, and stained with 0.5% Coomassie blue G-250 for 3 hours. The gels were digitized using a scanning digitizing system and analyzed using NIH image software. The MMP-2 (72-62 kDa) and MMP-9 (92-85 kDa) activities were normalized by MMP-2 standards concurrently run within the same gel to avoid the differences among gels, and the value for each Sham+AMD, MI, or MI+AMD was calculated as a ratio to that from MMP-2 standard.

Cardiac content of MMP-2

MMP-2 content in the non-infarct LV myocardium was also measured by a commercially available enzyme-linked immunosorbent assay (ELISA) according to the manufacture's instructions (MMP-2: Biotrak, GE Healthcare, UK).

Statistical analysis

All values are expressed as mean ± SEM. For multiple-group comparisons, ANOVA followed by post hoc analysis with Scheffé's test was performed. $P<0.05$ was considered statistically significant.
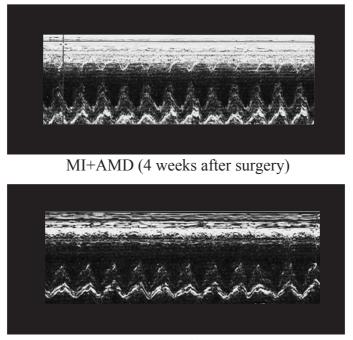
Results

Echocardiographic and hemodynamic measurements

Neither blood pressure nor heart rate was different among the 4 groups. There were no

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Table 1 Histomorphometric, Echocardiographic, and Hemodynamic Data

	Sham (n=9)	Sham+AMD (n=4)	MI (n=7)	MI+AMD (n=7)
Histomorphometric				
BW, g	517 ± 29	435 ± 46	510 ± 14	465 ± 31
Heart wt/BW, mg/g	2.6 ± 0.1	3.0 ± 0.2	2.8 ± 0.1	2.8 ± 0.2
Lung wt/BW, mg/g	3.2 ± 0.3	3.7 ± 0.2	3.1 ± 0.2	3.6 ± 0.3
Infarct size (% of heart wt)	0	0	14.8 ± 2.6	21.5 ± 4.0
Echocardiographic				
LVDd, mm	7.4 ± 0.2	7.1 ± 0.2	7.5 ± 0.3	7.0 ± 0.3
LVFS, %	39.6 ± 0.7	40.3 ± 0.8	35.0 ± 1.0*	39.8 ± 1.6
Heart rate, bpm	393 ± 13	359 ± 12	387 ± 15	343 ± 17
SBP, mmHg	147 ± 2.7	150 ± 8.2	154 ± 3.4	139 ± 3.4
DBP, mmHg	115 ± 3.3	115 ± 4.1	118 ± 3.6	106 ± 1.9

BW=body weight; wt=weight; LVDd=left ventricular end-diastolic diameter; LVFS=left ventricular fractional shortening; SBP=systolic blood pressure; DBP=diastolic blood pressure. *$P<0.05$ vs Sham and MI+AMD.

MI+AMD (4 weeks after surgery)

MI (4 weeks after surgery)

Figure 1 Representative M-mode echocardiograms recorded at 4 weeks after surgery in the rats with coronary artery ligation without (MI) and with amiodarone (MI+AMD).

differences in the body weight, heart weight, lung weight, and LVDd at 4 weeks after surgery (Table 1). In MI and MI+AMD rats, myocardial infarction was created with an infarct size of 14.8±2.6% and 21.5±4.0% of heart weight, respectively (*P*=NS between MI and MI+AMD).

Representative M-mode echocardiograms of MI and MI+AMD rats recorded at 4 weeks after surgery are shown in Figure 1. As compared with MI, the motion of the interventricular septum is preserved in MI+AMD. As shown in Figure 2, LVFS were similar between MI and MI+AMD rats at baseline and just before operation. At 4 weeks after surgery, LVFS was decreased in the
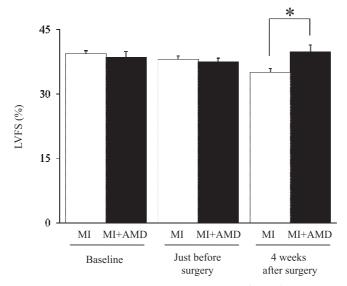
Figure 2 Comparison of the left ventricular fractional shortening (LVFS) at baseline, just before operation, and at 4 weeks after surgery between rats with coronary artery ligation without (MI) and with amiodarone (MI+AMD). *$P<0.05$ for difference from corresponding MI value.
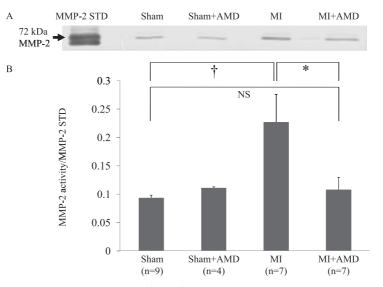

Figure 3 Comparison of matrix metalloproteinese (MMP)-2 activities measured by zymography among sham-operated (Sham, n=9), sham-operated with amiodarone treatment (Sham+AMD, n=4), left coronary artery (LCA)-ligated without amiodarone (MI, n=7) and LCA-ligated with amiodarone groups (MI+AMD, n=7). A, Representative gelatin zymograms for Sham, Sham+AMD, MI, and MI+AMD groups with MMP-2 standard (STD). B, Densitometric analysis of MMP-2 activities in Sham, Sham+AMD, MI, and MI+AMD groups. MMP-2 activities are expressed as a ratio to the MMP-2 STD. *$P<0.05$, † $P<0.01$ for difference from MI value.

MI rats but not in MI+AMD rats ($P<0.05$). Thus, in this experimental rat myocardial infarction model, amiodarone improved LV systolic dysfunction after myocardial infarction.

Effects of amiodarone on MMP activity and content in the LV myocardium

In gelatin zymography, the proteolytic activity of MMP-2 was detected at 72 kDa, corresponding to gelatinase A. MMP-2 activity was detected in all LV samples (n=27). MMP-9 was not detected in any of them. As show in Figure 3, amiodarone did not affect MMP-2 activity in Sham. MMP-2 activity was increased in MI rats compared
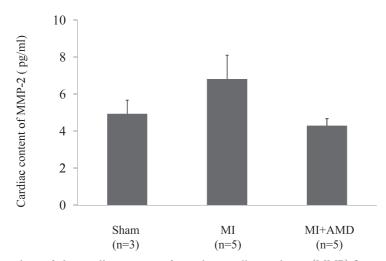
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Figure 4 Comparison of the cardiac content of matrix metalloproteinese（MMP)-2 among sham-operated
（Sham, n=3), left coronary artery（LCA)-ligated without amiodarone（MI, n=5) and LCA-ligated
with amiodarone groups（MI+AMD, n=5).

with that in Sham（0.23±0.05 versus 0.09±0.01, P<0.01). This increase in MMP-2 activity in MI was inhibited by amiodarone（P<0.05, MI+AMD versus MI; P=NS, MI+AMD versus Sham).

The cardiac content of MMP-2 was measured by ELISA. As shown in Figure 4, MMP-2 level was higher in MI rats than in Sham and MI+AMD rats, but the difference did not reach a statistical significance.

Discussion

Using a rat myocardial infarction model, the present study clearly showed that amiodarone suppresses the increase in the MMP-2 activity occurring in the non-infarct myocardium. This appears to be related to its improving effect on the ventricular function.

In this study, we administered 20 mg/kg of amiodarone daily to investigate amiodarone's effect on structural remodeling, and it may be pointed out that the dose used was relatively large. In the clinical setting, the initial dose of amiodarone recommended in Japan is 400 mg/day（approximately 8 mg/kg/day) for 14 days followed by 200 mg/day. In the Western countries, however, the initial dose commonly used is 600 to 800 mg/day followed by 400 mg/

day. In the previous experimental studies, the larger doses of amiodarone than the present one have been used. Hu et al. examined the effect of various doses of oral amiodarone on heart rate in the experimental rat myocardial infarction model and found no significant effect when the dose was 20 mg/kg/day.[26] The purpose of this study was not to investigate amiodarone's antiarrhythmic effect but to examine its effect on the process of the development of structural remodeling. We therefore decided to use the dose of 20 mg/kg/day for the experiment.

It may also be pointed out the reason why amiodarone was administered for the 2-week period before operation. After oral administration, amiodarone only has a bioavailability of 30％. It also has an extremely long plasma half-life of 19- 53 days, so it can take a long time to reach a therapeutic concentration and loading doses are therefore frequently used to accelerate this process in clinical practice. Although the present study could not exclude additional effects of amiodarone which were initiated before operation, we started to administer amiodarone 2 weeks before operation.

Many clinical studies have demonstrated the effectiveness of amiodarone against both

supraventricular and ventricular tachyarrhythmias compared with the other antiarrhythmic drugs. Roy et al. reported the superiority of amiodarone in preventing the recurrence of atrial fibrillation to propafenone, a class I antiarrhythmic drug, and sotalol, a class III drug.[27] In the substudy of EMIAT, it was reported that the patients with left ventricular ejection fraction ≤40% and depressed heart rate variability benefit from the prophylactic treatment with amiodarone.[28] Unlike the other antiarrhythmic drugs, amiodarone was said not to depress ventricular function, and was recommended for the patients with symptomatic tachyarrhythmias and with heart failure. In CHF-STAT, amiodarone was shown not to worsen the prognosis of the patients with congestive heart failure and left ventricular ejection fraction ≤40%.[4] These suggest that amiodarone has some potentials other than its antiarrhythmic drug properties.

We recently reported that amiodarone suppresses both electrical and structural remodeling in a canine pacing-induced persistent atrial fibrillation model.[22] In addition to its prolonging effect on the effective refractory period and reversing effect on the impaired rate-adaptation of refractoriness induced by continuous rapid atrial pacing, we found that amiodarone decreased the extent of the interstitial fibrosis in the left atrium. To elucidate this amiodarone's effect on atrial structural remodeling, we further examined its effect on MMP-2 activity and found that amiodarone decreased the enhanced MMP-2 activity in the "fibrillating" left atrium to the control level. Thus, amiodarone may have an attenuating effect on MMP activity, but it still remains to be established. Using a mouse acute myocardial infarction model, Hayashidani et al. showed that MMP-2 activity was enhanced in the non-infarct myocardial tissue, and fluvastatin suppressed this enhanced enzyme activity.[29] We used a rat myocardial infarction model to confirm the attenuating effect of amiodarone on MMP

activity induced by acute myocardial infarction. The results clearly showed the suppressing effect of amiodarone on MMP-2 activity enhanced in the MI rats. The study further showed the improving effect of amiodarone on the left ventricular function. It is unclear whether the decreased MMP activity is linked to the improved ventricular function. The suppression of MMP with amiodarone may inhibit the development of ventricular remodeling. A further study is necessary.

The mechanism for the attenuating effect of amiodarone on MMP activity could not be clarified in this study. It was reported that mechanical stretch induces reactive oxygen species formation via the NAD(P)H oxidase and enhances MMP-2 mRNA expression and pro-MMP-2 release.[30] Amiodarone was shown to protect cardiac myocytes against oxidative stress-mediated injury by directly scavenging oxygen free radical.[12] Amiodarone may suppress the MMP-2 activity via its free radical scavenging action. Tumor necrosis factor (TNF)-α is one of the cytokines that can induce transcription of MMP.[31] It was reported that amiodarone suppresses inflammatory cytokines including TNF-α and interleukin-6.[10] Furthermore, mast cell chymase has been reported to regulate pro-MMP-2 and -9 activities.[32, 33] Several studies demonstrated that cardiac mast cell degranulation mediates MMP activation and extracellular matrix degradation.[34−36] It was also reported that amiodarone inhibited the proliferation of mast cells by arresting them in the G2 phase of the cell cycle.[15] These amiodarone's effect on the oxidative stress, inflammatory cytokines and mast cells may be related to the present amiodarone's effect on MMP activity. Further biochemistric studies on amiodarone's effects on MMP should be required.

Conclusions

Amiodarone exerts an inhibitory effect on the MMP-2 activity. This may be related to the

improvement of left ventricular function in MI rats.

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