Chymase Plays an Important Role in Left Ventricular Remodeling Induced by Intermittent Hypoxia in Mice

Chika Matsumoto, Tetsuya Hayashi, Kento Kitada, Chika Yamashita, Masatoshi Miyamura, Tatsuhiko Mori, Akira Ukimura, Mamoru Ohkita, Denan Jin, Shinji Takai, Mizuo Miyazaki, Yoshikatsu Okada, Yasushi Kitaura, Yasuo Matsumura

Abstract—Intermittent hypoxia caused by sleep apnea is associated with cardiovascular disease. Chymase has been reported to play an important role in the development of cardiovascular disease, but it is unclear whether chymase is involved in the pathogenesis of left ventricular remodeling induced by intermittent hypoxia. The aim of this study was to evaluate the effect of a novel chymase inhibitor (NK3201) on hypoxia-induced left ventricular remodeling in mice. Male C57BL/6J mice (9 weeks old) were exposed to intermittent hypoxia or normoxia and were treated with NK3201 (10 mg/kg per day) or the vehicle for 10 days. Left ventricular systolic pressure showed no significant differences among all of the experimental groups. Exposure to intermittent hypoxia increased left ventricular chymase activity and angiotensin II expression, which were both suppressed by treatment with NK3201. Intermittent hypoxia also increased the mean cardiomyocyte diameter, perivascular fibrosis, expression of inflammatory cytokines, oxidative stress, and NADPH-dependent superoxide production in the left ventricular myocardium. These changes were all suppressed by NK3201 treatment. Therefore, chymase might play an important role in intermittent hypoxia-induced left ventricular remodeling, which is independent of the systemic blood pressure. (Hypertension. 2009;54:00-00.)

Key Words: sleep apnea ▪ hypoxia ▪ chymase ▪ cardiac remodeling ▪ oxidative stress

Sleep apnea syndrome (SAS) is an important risk factor for several cardiovascular diseases.1 In addition, intermittent hypoxia attributed to SAS has been suggested to have a role in the development of left ventricular (LV) remodeling.2,3 Oxidative stress, inflammatory cytokines, and transforming growth factor-β (TGF-β) have been reported to play important roles in the progression of LV remodeling,4 which is accompanied by hypertrophy of cardiomyocytes and an increase of interstitial fibrosis. Plasma levels of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) are elevated in patients with SAS,5,6 indicating a relationship between the inflammatory response and intermittent hypoxia.

Chymase is one of the enzymes that produces angiotensin II (Ang II) and is a chymotrypsin-like serine protease that is abundant in the secretory granules of mast cells.7 In patients with heart failure, the cardiac mast cell density is markedly increased, and chymase might play a role in the development of several cardiovascular diseases.8,9 Chymase usually exists as an inactive form in the secretory granules of mast cells, and it is activated after release in injured tissues.10–12 Therefore, chymase inhibitors may suppress Ang II production by activated chymase in injured tissues and may exert this pharmacological action.

SAS patients are known to have high plasma levels of Ang II and aldosterone,13 and hypoxic stress has been shown to increase the circulating level of Ang II.14 We reported previously that hypoxic stress induced LV remodeling and increased oxidative stress in mice, whereas these changes were suppressed by angiotensin receptor blocker therapy.15 Therefore, an increase of Ang II expression because of intermittent hypoxic stress seems to promote cardiac remodeling by acceleration of oxidative stress. In addition, cardiac chymase promotes fibrosis by activating the production of TGF-β16,17 and inflammatory cytokines.18 In the present study, we evaluated the role of chymase in the development of LV remodeling attributed to intermittent hypoxia in mice, using a specific chymase inhibitor (NK3201) that inhibits chymase in a competitive and reversible fashion.19–22

Methods

Drug
The specific chymase inhibitor 2-[5-formylamino-6-oxo-2-phenyl-1,6-dihydropyrimidine-1-yl)-N-[(3,4-dioxo-1-phenyl-7-[2-pyridyloxy])-2-heptyl] acetamide (NK3201; Nippon Kayaku) was synthesized.21

Animals
Male C57BL/6J mice were used at 9 weeks of age. The animals were housed in a room with a 12-hour light/dark cycle and were allowed free access to food and water. The experimental protocol and
methods of animal care during the experiments were approved by the experimental animal research committee of Osaka University of Pharmaceutical Sciences, and all of the studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Experimental Protocol
Mice were divided into 2 groups and were placed into chambers that delivered intermittent hypoxia (30 seconds of 4.5% to 5.5% O₂, followed by 30 seconds of 21% O₂ for 8 h/d during the daytime) or were housed under normoxic conditions for 10 consecutive days. The duration and severity of intermittent hypoxia were determined from our previous study. The hypoxic and normoxic animals were divided into 2 groups, each of which were treated with vehicle or the chymase inhibitor NK3201 (10 mg/kg per day, PO). As we have reported, that treatment with a dose from 1 to 30 mg/kg of NK3201 is useful for chymase inhibition, we chose the dose of 10 mg/kg per day in the present study.

After the 10-day experimental period, cardiac catheterization was done under IP anesthesia with pentobarbital sodium (40 mg/kg). The right ventricular systolic pressure (RVSP) and LV systolic pressure were measured by the method published previously. After blood sampling for the measurement of lipid peroxide (LPO), the heart was excised, and the upper half was used for light microscopic examination. The free wall of the LV myocardium was excised for sampling for the measurement of lipid peroxide (LPO), the heart was excised, and the upper half was used for light microscopic examination. The shortest diameter of each nucleated cardiomyocyte was measured by the method published previously. After staining with Sirius red, color images were taken of 5 randomly selected high-power fields (×200) on 5 sections per animal, and the percentage of collagen volume was calculated. Sections stained with trudine blue were obtained to evaluate mast cell infiltration, and fine structure was examined under an electron microscope (model H-7650, Hitachi).

Enzyme Activity
LV extracts for measurement of chymase and ACE activities were prepared as described previously. Chymase and ACE activities were measured using synthetic substrates, Suc-Ala-Ala-Pro-Phe-4-methylcoumaryl-7-amide and hippuryl-His-Leu, respectively. Protein concentrations were assayed by the method published previously. A polyclonal antibody targeting Ang II (IgG Corp) was used, and Ang II concentration in the final reaction mixture was 5 μmol/L, and NADPH-dependent superoxide production was measured as relative light units per minute per milligram of protein.

Table. Effect of Hypoxia on Hemodynamic Parameters and Enzyme Activities

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normoxia, Mean ± SEM (n)</th>
<th>Hypoxia, Mean ± SEM (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW, g</td>
<td>Vehicle</td>
<td>NK3201</td>
</tr>
<tr>
<td></td>
<td>24.6 ± 0.9 (9)</td>
<td>24.9 ± 0.5 (12)</td>
</tr>
<tr>
<td></td>
<td>22.7 ± 0.7 (10)</td>
<td>23.6 ± 0.3 (9)</td>
</tr>
<tr>
<td>HW, mg</td>
<td>93.3 ± 4.4 (14)</td>
<td>101.9 ± 4.4 (12)</td>
</tr>
<tr>
<td></td>
<td>106.3 ± 3.6 (10)</td>
<td>94.4 ± 3.3 (8)</td>
</tr>
<tr>
<td>HW:BW, mg/g</td>
<td>4.0 ± 0.1 (9)</td>
<td>4.1 ± 0.2 (12)</td>
</tr>
<tr>
<td></td>
<td>4.7 ± 0.2 (10)†</td>
<td>4.0 ± 0.2 (8)†</td>
</tr>
<tr>
<td>Hemodynamic data</td>
<td>RVSP, mm Hg</td>
<td>24.7 ± 1.4 (10)</td>
</tr>
<tr>
<td></td>
<td>27.1 ± 2.1 (8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>36.9 ± 5.0 (7)*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>29.4 ± 3.0 (8)†</td>
<td></td>
</tr>
<tr>
<td>LVSP, mm Hg</td>
<td>100.1 ± 1.9 (10)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>101.8 ± 1.1 (8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>104.4 ± 3.2 (7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100.7 ± 2.2 (6)</td>
<td></td>
</tr>
<tr>
<td>Enzyme activities</td>
<td>Chymase activity, mU/mg protein</td>
<td>1.0 ± 0.1 (9)</td>
</tr>
<tr>
<td></td>
<td>0.98 ± 0.07 (7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.5 ± 0.25 (8)*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.87 ± 0.03 (10)†</td>
<td></td>
</tr>
<tr>
<td>ACE activity</td>
<td>0.39 ± 0.1 (7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.47 ± 0.05 (7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.92 ± 0.2 (7)*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.71 ± 0.05 (9)*</td>
<td></td>
</tr>
</tbody>
</table>

LVSP indicates LV systolic pressure. *P < 0.05 vs normoxic mice treated with vehicle. †P < 0.05 vs hypoxic mice treated with vehicle.

Immunohistochemistry for 4-Hydroxy-2-Nonenal and Ang II
The sections cut from the paraffin blocks were incubated with a monoclonal antibody directed against 4-hydroxy-2-nonenal (4-HNE; No. MHN-20, Japan Institute for the Control of Aging) or with a polyclonal antibody targeting Ang II (IgG Corp). The percentage area of 4-HNE and Ang II staining was measured by the method reported previously.

NADPH Oxidase Activity
NADPH-dependent superoxide production was measured by a lucigenin-enhanced chemiluminescence assay. The lucigenin concentration in the final reaction mixture was 5 μmol/L, and NADPH-dependent superoxide production was expressed as relative light units per minute per milligram of protein.

Quantitative Real-Time Reverse-Transcription PCR
Total RNA was extracted from heart tissues with an RNeasy Mini Kit (Qiagen). Real-time quantitative PCR was done by the method published previously. The amount of target mRNA obtained was evaluated by comparison with GAPDH mRNA expression.

Statistical Analysis
Data were expressed as the mean ± SEM. Statistical analysis was done by using 1-way ANOVA, followed by Fisher’s protected least significant difference multiple comparison tests. Significance was recognized at P < 0.05.

Results
Heart and Body Weights
Intermittent hypoxic stress tended to decrease the body weight (BW) and increase the heart weight (HW), leading to an increase of the HW:BW ratio, whereas this change was significantly suppressed by treatment with NK3201 (Table).

Hemodynamic Measurements
RVSP was significantly increased in mice exposed to intermittent hypoxia, whereas this change was suppressed by NK3201. There were no significant differences in LV systolic pressure among the 4 groups (Table).

Enzyme Activity
Intermittent hypoxic stress significantly increased both chymase and ACE activities in LV myocardium. Treatment with NK3201 significantly suppressed chymase activity but not...
ACE activity. Chymase and ACE activities in the normoxic group were not altered by NK3201 treatment (Table).

**Histological Findings**

Intermittent hypoxic stress caused disarrangement of myofibers and hypertrophy of cardiomyocytes. The mean cardiomyocyte diameter was significantly increased in mice exposed to intermittent hypoxia, whereas this change was suppressed by treatment with NK3201 (Figure 1). Perivascular fibrosis was significantly increased in the LV myocardium of mice exposed to intermittent hypoxic stress, which was significantly suppressed by treatment with NK3201 (Figure 2). Infiltration of mast cells was observed in the vicinity of small arteries of LV myocardium. Hypertrophy of the smooth muscle of small arteries was also increased in mice exposed to intermittent hypoxic stress (Figure 3). None of these degenerative changes induced by intermittent hypoxic stress were observed in normoxic mice or NK3201-treated mice.

**Ang II Expression**

Mild positive staining with Ang II antibody was observed in the coronary artery walls and in some of the cardiomyocytes of normoxic mice. Intermittent hypoxic stress led to an increase of Ang II expression in vascular smooth muscle cells and the cytoplasm of cardiomyocytes near the coronary arteries. These changes were significantly suppressed by treatment with NK3201 (Figure 4).

**NADPH-Dependent Superoxide Production**

NADPH-dependent superoxide production in the LV myocardium was significantly increased by exposure to intermittent hypoxia, whereas this change was significantly suppressed by treatment with NK3201 (Figure 5).

**Plasma LPO and 4-HNE Expression**

The plasma LPO level was significantly elevated in mice exposed to intermittent hypoxia. This change was suppressed by treatment with NK3201 (Figure 5). Expression of 4-HNE protein, a specific product of lipid peroxidation and a marker of oxidative stress, was significantly increased in the LV myocardium by intermittent hypoxia. Treatment with NK3201 led to a significant decrease of 4-HNE protein expression (Figure 6).

**Reverse-Transcription PCR**

Intermittent hypoxic stress significantly increased TNF-α, IL-6, and TGF-β mRNA expression in the LV myocardium.
Expression of IL-6 and TGF-β mRNA was significantly reduced by NK3201 treatment, whereas TNF-α mRNA expression tended to decrease (Figure 7).

Discussion

Exposure to intermittent hypoxia for 10 days significantly increased RVSP, although there was no significant change in LV systolic pressure in any of the groups. We have reported previously that the effect of pulmonary hypertension on LV remodeling was minimal in animals exposed to continuous hypoxia for 3 weeks. Therefore, the elevation of RVSP observed in this study probably had little influence on LV remodeling.

It has been reported that chymase inhibitors have a weaker antihypertensive effect than ACE inhibitors. NK3201 inhibits chymase activity by an IC50 at concentrations of 2.5, 1.2, and 28.0 mmol/L, respectively, but ACE activity was not inhibited by concentrations ≤100 μmol/L of NK3201. In the present study, NK3201 had no effect on blood pressure, because chymase was not activated in the plasma or because Ang II production by chymase in the vasculature was heterogeneous because of the short duration of our experiment. Monitoring the blood pressure during exposure to hypoxia and observation for a longer period should be performed in future studies.

Recently, several studies have suggested the involvement of chymase in the development of LV remodeling. In the present study, perivascular fibrosis was markedly aug-

Figure 3. Representative light micrographs stained with truidine blue (A through C) and electron micrographs of LV myocardium (D through F). A and D, Normal small artery in the myocardium of a normoxic mouse. B and F, Mononuclear cell with granules (possible a mast cell) in the vicinity of small artery in a mouse exposed to intermittent hypoxia. E, Hypertrophic smooth muscle cell (SMC) of a small artery (endothelial cell; EC) in a mouse exposed to intermittent hypoxia. C, No mast cell is observed in mice treated with NK3201.

Figure 4. Immunohistochemistry (A) and percentage area (B) of Ang II in LV myocardium. Ang II expression was significantly increased by intermittent hypoxia, which was suppressed by NK3201. Columns and bars represent the mean±SEM (n=5 to 9). Preabsorption of the antibody with Ang II abolished any positive reaction (negative control).
mented in mice exposed to intermittent hypoxia, which was significantly suppressed by NK3201. Furthermore, NK3201 treatment normalized the increase of the HW:BW ratio and the mean cardiomyocyte diameter in mice exposed to intermittent hypoxia. We have not directly compared the presence and localization of chymase in the heart of mice with humans and other animals, so further examination should be planned.

SAS patients are known to have increased plasma levels of Ang II, and hypoxic stress has also been shown to increase Ang II. In the present study, Ang II expression and chymase and ACE activities were increased in the LV myocardium by exposure to intermittent hypoxia. Moreover, we confirmed that treatment with NK3201 reduced chymase activity and cardiac Ang II levels but not ACE activity. Thus, it might be that the beneficial effect of NK3201 on LV remodeling was at least partly attributed to a decrease of Ang II expression via inhibition of chymase activity in the LV myocardium. Evaluation of other enzymes, including cathepsin, might be the next issue in our experiment.

Expression of TNF-α and IL-6 mRNA in the LV myocardium was significantly increased by exposure to intermittent hypoxia, which was suppressed by NK3201. It has been suggested that chymase has a role in inflammation and induces inflammatory cell accumulation, whereas NK3201 has been reported to show an anti-inflammatory effect. Moreover, cardiac overexpression of TNF-α might cause LV remodeling, whereas a chymase inhibitor suppresses both LV remodeling and inflammation. Taken together with our results, the reduction of inflammatory cytokines by NK3201 treatment might be closely related to the suppression of LV remodeling.

Figure 5. A, Effect of intermittent hypoxic stress on plasma LPO levels. B, NADPH-dependent superoxide production. LPO and NADPH-dependent superoxide production were significantly increased in mice exposed to intermittent hypoxia. They were normalized by treatment with NK3201. Columns and bars represent the mean±SEM (n=5 to 10).

Figure 6. Immunohistochemistry (A) and percentage area (B) of 4-HNE in LV myocardium. The expression of 4-HNE was significantly increased in mice exposed to intermittent hypoxia, which was normalized by treatment with NK3201. Columns and bars represent the mean±SEM (n=4 to 6).
TGF-β has a major role in the tissue fibroinflammatory response. It has been reported that overexpression of TGF-β enhanced extracellular matrix protein synthesis and led to cardiomyocyte hypertrophy. In the present study, expression of TGF-β mRNA in the LV myocardium was significantly increased by exposure to intermittent hypoxia, which was significantly suppressed by treatment with NK3201. Furthermore, intermittent hypoxia caused LV remodeling that was characterized by perivascular fibrosis and cardiomyocyte hypertrophy, whereas NK3201 treatment led to significant improvement of LV remodeling. Thus, chymase might play an important role in the development of intermittent hypoxia-induced LV remodeling via TGF-β.

Chymase is released from mast cells when tissue damage occurs, and mast cell degranulation is also stimulated in chronic inflammatory states, thus releasing various inflammatory mediators that include chymase. Infiltration and proliferation of mast cells have been reported to increase in failing hearts. In addition, TNF-α released from cardiac mast cells plays a crucial role in inducing IL-6 expression. Mast cells also store TGF-β in intracellular granules and secrete it to be activated by chymase. In the present study, infiltration of mast cells in the vicinity of small arteries of LV myocardium was observed, although the actual number of cells was small. It is possible that intermittent hypoxia might lead to the accumulation of mast cells, followed by increased secretion of chymase and accelerated LV remodeling. However, further studies, including quantitative analysis, need to be done, with a focus on the role of mast cells in hypoxia-induced LV remodeling.

Oxidative stress is a potent stimulus of inflammation and TGF-β overexpression and is involved in LV remodeling by contributing to hypertrophy, apoptosis, and fibrosis. Recently, it was reported that inhibition of superoxide dismutase induced collagen production in cardiac fibroblasts. Additional studies might clarify these discordant findings in the near future.

An increase of oxidative stress has been reported in patients and animals with heart failure. In the present study, the plasma LPO level, a marker of systemic oxidative stress, was significantly increased in mice exposed to intermittent hypoxia. In addition, intermittent hypoxia significantly enhanced NADPH-dependent superoxide production and 4-HNE expression in the LV myocardium. Treatment with NK3201 reduced oxidative stress, Ang II expression, inflammatory cytokine levels, and TGF-β mRNA expression in the LV myocardium and consequently suppressed the induction of LV remodeling by intermittent hypoxia. Thus, it can be suggested that intermittent hypoxia promotes oxidative stress via Ang II overexpression by activation of chymase, leading to the upregulation of inflammatory cytokines and TGF-β, resulting in the development of LV remodeling. The precise mechanisms by which chymase causes oxidative stress still need to be elucidated.

In conclusion, chymase plays a crucial role in intermittent hypoxia-induced LV remodeling by increasing oxidative stress, at least partly through the upregulated expression of Ang II and inflammatory cytokines. Treatment with NK3201 might be potentially useful for preventing cardiovascular events in patients with SAS.

Perspectives

We demonstrated that a selective chymase inhibitor NK3201 improved intermittent hypoxia-induced LV remodeling, independent of any effect on systemic blood pressure. The beneficial effect of NK3201 might be related to a decrease of Ang II expression by suppressing chymase activation in injured tissue. All of the patients with SAS do not have accompanying hypertension. Therefore, chymase inhibition, unlike an angiotensin receptor blocker, could suppress only local Ang II via the chymase pathway, and this targeted inhibition of local Ang II might be a merit. Additional studies are required to compare the effect of chymase inhibition on intermittent hypoxia-induced LV remodeling with the effects of an ACE inhibitor and angiotensin receptor blocker.

Acknowledgments

We express our gratitude to Sadao Uchida, Chieko Ota, Yumiko Ogami, Minako Ito, Motoyo Kobayashi, Yasuko Mizuoka, Yoshiro Kitaguni, and Teruo Ueno (Central Research Laboratory, Osaka Medical College) for their expert technical assistance, and we also acknowledge the secretarial assistance of Fusako Maeda and Junko Yoshimura during preparation of this article.

Sources of Funding

This work was supported by Kakenhi (2051092), a Grant-in-Aid for Scientific Research (C), from the Ministry of Education, Culture, Sports,
Disclosures

None.

References

Chymase Plays an Important Role in Left Ventricular Remodeling Induced by Intermittent Hypoxia in Mice
Chika Matsumoto, Tetsuya Hayashi, Kento Kitada, Chika Yamashita, Masatoshi Miyamura, Tatsuhiko Mori, Akira Ukimura, Mamoru Ohkita, Denan Jin, Shinji Takai, Mizuo Miyazaki, Yoshikatsu Okada, Yasushi Kitaura and Yasuo Matsumura

Hypertension. published online May 26, 2009;
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/early/2009/05/26/HYPERTENSIONAHA.109.131391.citation

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/