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# Heme Oxygenase-1 Reduces Murine Monocrotaline-Induced Pulmonary Inflammatory Responses and Resultant Right Ventricular Overload

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## ABSTRACT

**Monocrotaline (MT), a pyrrolizidine alkaloid, causes pulmonary hypertension (PH) in rats and is widely utilized to analyze the pathophysiology of PH. However, a murine PH model with which transgenic animals may be used has not been established. To establish a murine MT-induced PH model, we administered different amounts of MT and determined the extent of right ventricular (RV) overload and PH. We also examined the expression of heme oxygenase-1 (HO-1), a potential antistress protein in MT-treated animals, and evaluated the functional role of HO-1 by administering an HO-1 inhibitor. Significant pulmonary inflammation and RV hypertrophy were observed when mice were given 600 mg/kg weight of MT weekly for 8 weeks. In addition, elevated RV pressure and induction of HO-1 in lung and RV were observed with this dose of MT. Interestingly, inhibition of HO activity promoted inflammatory changes in the lung and the resultant RV hypertrophy. HO-1 may play defensive roles against murine MT-induced pulmonary inflammation and the resultant RV overload.** *Antioxid. Redox Signal.* 4, 563–568.

## INTRODUCTION

THE PYRROLIZIDINE ALKALOID MONOCROTALINE (MT) is a phytotoxin used experimentally to cause in rats (3, 11), and dogs (20) a pulmonary vascular syndrome characterized by proliferative pulmonary vasculitis, pulmonary hypertension (PH), and right ventricular (RV) hypertrophy and resulting in death. However, there have been few MT-induced PH models in mice (8, 22), an organism amenable to genetic manipulation.

Microsomal heme oxygenase (HO) catalyzes the degradation of heme into equimolar amounts of biliverdin, carbon monoxide (CO), and free iron (18, 35). Recent studies suggest that, in addition to its function in heme degradation (35), this enzyme may protect against oxidative stresses (31, 38) and atherosclerosis (14) and help maintain vascular tone (5, 34). It has also been reported that HO-1, an inducible form of HO, is expressed in the heart of rats subjected to ischemia/reperfusion injury (7, 19, 24) and in cultured cardiomyocytes under hypoxic conditions (12). HO-1 is also induced in the pressure-overloaded heart, such as that in the pulmonary artery banding model (15). The functional significance of HO-1 induction under these conditions, however, is not clear.

In the present study, we administered different amounts of MT to mice and examined the extent of RV overload and PH to establish a murine model of MT-induced PH (4). We also examined the expression of HO-1, as induction of this enzyme may represent an antistress response in MT-treated animals, and evaluated the protective role of HO-1 by administering an HO-1 inhibitor. Treatment with large amounts of MT produced prominent inflammatory changes in the lung, PH, and RV hypertrophy with HO-1 induction. Mice treated with the HO inhibitor exhibited more severe pathological changes than untreated animals. These data suggest that HO-1 induction represents an intrinsic defense system during MT-induced pulmonary inflammation and cardiac hypertrophy.

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## MATERIALS AND METHODS

### *Animal handling and procedures*

Three-week-old male C57BL/6J mice were used in this study (CLEA Japan, Inc, Tokyo, Japan). Animals were challenged with different amounts of MT (Wako Pure Chemical, Osaka, Japan) by subcutaneuous injection weekly for 8 weeks. Sn-protoporphyrin IX (SnPP IX; 30 mg/kg body weight; Sn group) or saline (C group) was administered three times per week. Body weight (BW) was measured before and after the pharmacological intervention. After 8 weeks, animals were anesthetized and killed by cervical dislocation. The heart was isolated, divided into RV and left ventricle with the interventricular septum (LV), and the wet weights of RV and LV were measured. The wet weights of both lungs were also measured.

### *Measurement of RV pressure*

Mice were anesthetized by intraperitoneal injection of sodium pentobarbital (50 μg/g body weight), intubated with a 22 G tube, and ventilated with a Harvard rodent ventilator (Model 683, Harvard Apparatus, Holiston, MA, U.S.A.). After a left-parasternal incision, small parts of the ribs were resected and the pericardium was opened. A Mikro-Tip catheter transducer (Model SPR-671, Millar Instruments, Inc., Houston, TX, U.S.A.) was inserted directly into the RV, and RV pressure was monitored.

### *Histological analyses of RV and lung*

For histological analyses, heart and lung tissues were washed briefly with saline and fixed with 4% paraformaldehyde. Five-micrometer serial sections were prepared and stained with hematoxylin–eosin. To evaluate hypertrophied changes of RV cardiomyocytes, mean transverse diameters ($n$ = 200, each group) were measured under light microscopy (Model AX80T, Olympus, Tokyo, Japan). Pulmonary arterial thickening was examined under light microscopy and quantified by calculating the percent pulmonary artery thickness (%PAT) with the NIH image software, using the following equation: %PAT = 100 × (perivascular area − luminal area)/luminal area. For the %PAT calculation, 40 arteries of <50 μm were analyzed.

### *RNA extraction and northern blot analysis*

Total RNA was isolated from RV, LV, and lung tissues using Trizol reagent (GibcoBRL). Fifteen micrograms of total RNA was electrophoresed in a formaldehyde/1% agarose gel, transferred to a nylon membrane, and cross-linked by UV irradiation. The blots were prehybridized, hybridized with $^{32}$P-labeled rat HO-1 cDNA, washed, and exposed to Hyperfilm-ECL (Amersham Pharmacia Biotech, Uppsala, Sweden) as previously described (13).

### *Western blot analysis*

For western blot analysis of HO-1 expression, 20 μg of lung and RV tissue homogenates were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and the proteins were blotted onto polyvinylidene difluoride (PVDF) membranes (ATTO, Tokyo, Japan). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 and incubated for 1 h at 37°C with polyclonal antibody against HO-1 (StressGen, Vancouver, BC, Canada). HO-1 was detected using the ECL chemiluminescent detection system (Amersham Pharmacia Biotech).

### *Data analysis*

All values are expressed as means ± SD. Differences were evaluated for significance by one-way ANOVA analysis. The Spearman correlation followed by $t$ test was used for the analysis of %PAT.

## RESULTS

### *Development of murine pulmonary hypertension induced by MT*

We first determined whether murine PH is induced by the administration of 60 mg/kg body weight of MT, which elicits PH in rats ($n$ = 10). This amount of MT, however, did not induce significant changes in lung and heart (data not shown). We increased the amount of MT to 300 mg/kg (300 MT group) and 600 mg/kg (600 MT group) and changed from a single injection of MT to weekly injections for 8 weeks ($n$ = 10, each group). Extensive infiltration of inflammatory cells in the lung interstitium, fibrotic changes, and pulmonary arterial thickening were observed in the 600 MT group (Fig. 1A and C). In addition, direct measurement of RV pressure showed remarkable elevation in the 600 MT group (Fig. 1B).

Mice treated with 600 MT exhibited significant lower BW compared with controls after 8 weeks (Fig. 2A). In addition, the ratios of RV weight to BW, RV weight to LV plus RV weight, and that of lung weight to BW were increased in the 600 MT group compared with controls (Fig. 2B, D, and E, respectively), whereas the ratio of LV weight to BW was similar (Fig. 2C). Microscopic hypertrophic changes in RV determined by the measurement of mean transverse diameter of cardiomyocytes was also observed in the 600 MT group (Fig. 3).

### *HO-1 induction in lung and hypertrophied RV in the MT-induced PH model*

We next determined whether HO-1, a stress-responsive protein (7, 10, 12–17), was induced in the RV myocardium of MT-treated mice. HO-1 protein was faintly expressed in the lung and RV of control, 60 MT-, and 300 MT-treated mice (Fig. 4A and B). In contrast, HO-1 was induced in 600 MT-treated mice, which showed significant inflammatory changes in the lung (Fig. 4A) and hypertrophied changes in the RV (Fig. 4B). The induction of HO-1 mRNA in the RV of 600 MT-treated mice was also confirmed by northern blot analysis (Fig. 4C).

To examine the function of HO-1 induction in this model, we treated animals with a specific inhibitor of HO, SnPP IX, during the 8 weeks of 600 MT treatment ($n$ = 10, 600 MT +

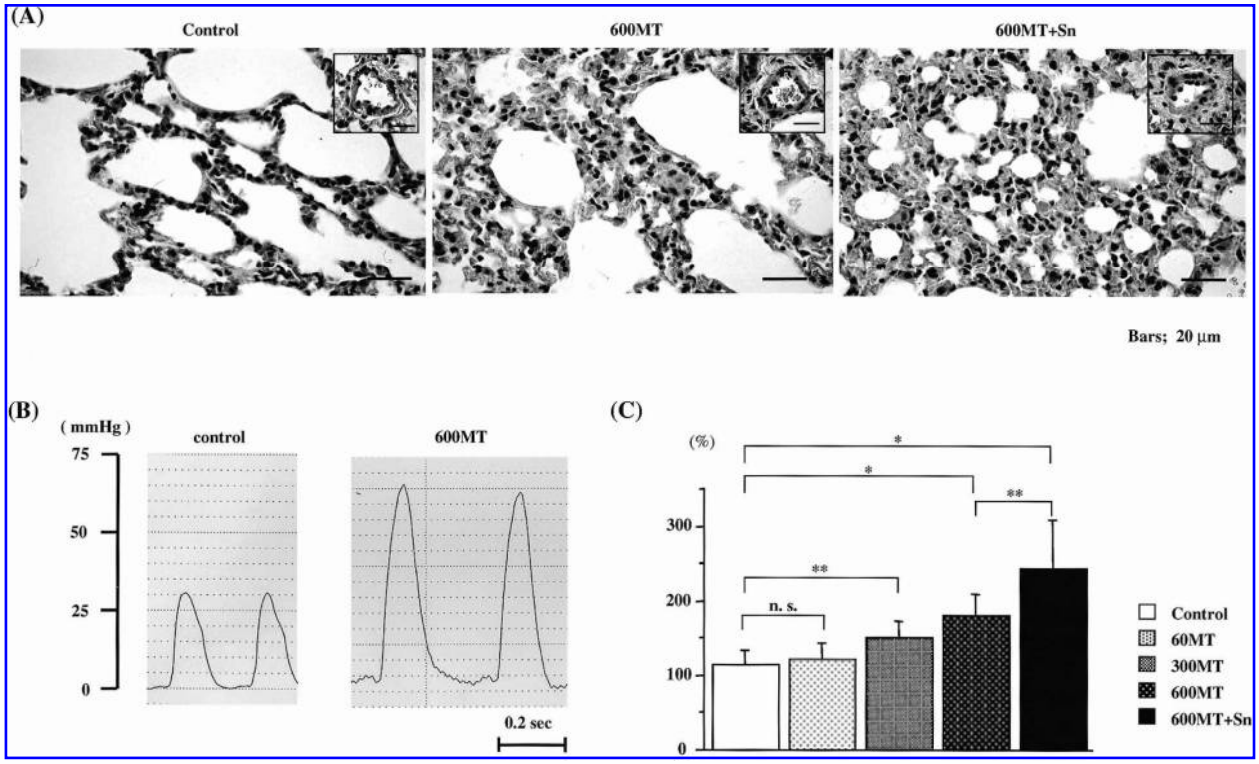


**FIG. 1.** **(A)** Photomicrographs of lung tissues after MT treatment with or without inhibition of HO. **Insets:** Pulmonary arteries. Bars = 20 μm. Hematoxylin–eosin staining. **(B)** Representative records of RV pressure curves. **(C)** Mean pulmonary artery thickening (%PAT). 60 MT, 300 MT, and 600 MT: 60, 300, and 600 mg/kg body weight administration of MT administered weekly ($n$ = 10, each group). 600 MT + Sn; HO-1 was inhibited by SnPP IX (30 mg/kg) injection of the 600 MT-treated mice ($n$ = 10). Data represent means ± SD. Asterisks represent statistically significant differences (*$p < 0.001$, **$p < 0.05$). n.s., not significant.

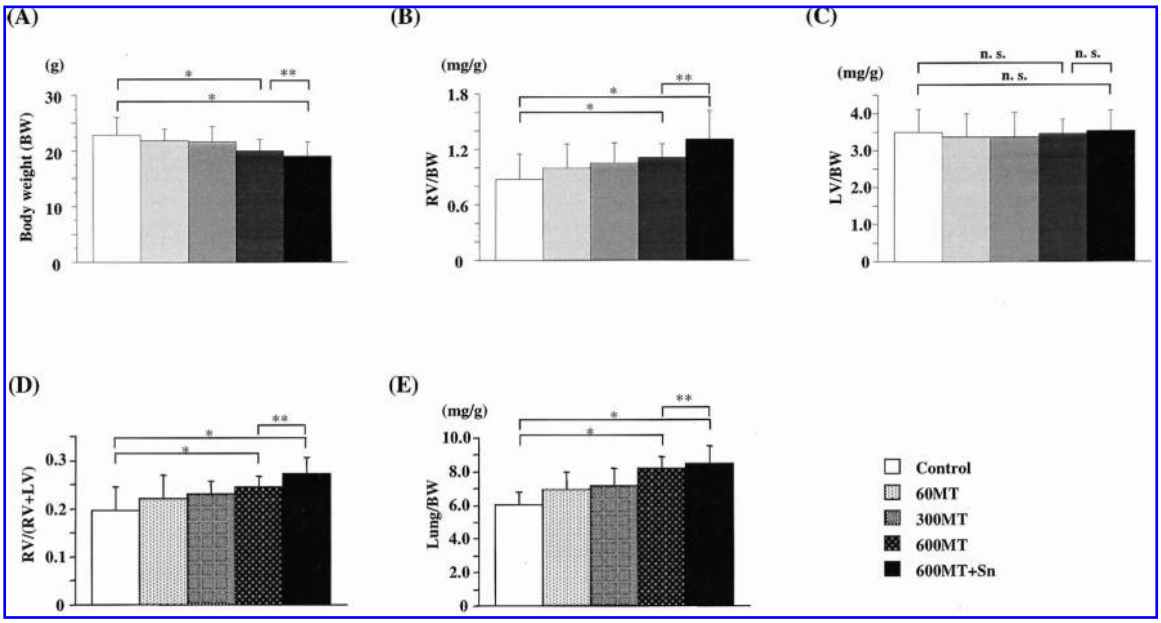


**FIG. 2.** **(A)** BW after administration of different amounts of MT for 8 weeks. **(B)** The ratio of RV weight/BW. **(C)** The ratio of LV weight/BW. **(D)** The ratio of RV weight/(RV + LV) weight. **(E)** The ratio of lung weight/BW. Data represent means ± SD (*$p < 0.001$, **$p < 0.05$). n.s., not significant.

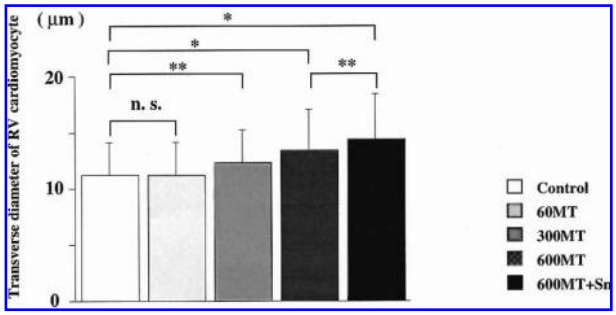


**FIG. 3. Changes in mean transverse diameter of RV cardiomyocytes (μm).** Transverse diameters of 200 RV cardiomyocytes were measured in each group. Data represent means ± SD (*$p < 0.001$, **$p < 0.05$). n.s., not significant.

Sn group). HO inhibition resulted in the promotion of inflammatory changes in lung and an increase in lung weight (Figs. 1A and 2E). Furthermore, HO inhibition promoted the increase of RV hypertrophy (Figs. 2B and D, 3). These results suggest that HO enzymatic activity provides antiinflammatory actions against MT-induced pulmonary inflammation and that induction of HO-1 in the RV itself may suppress myocardial hypertrophy.

## DISCUSSION

In this study, we established a murine model of MT-induced PH and RV hypertrophy by weekly administration of 600 mg/kg MT for 8 weeks. The dose of MT given the mice in this study is 80-fold higher than that used in conventional rat models (1, 3, 11, 21, 25). This requirement may be due to a lower amount of liver enzymes that convert MT to its active metabolites in mice than in rats (16, 32, 39). This difference would explain the difficulty in developing murine models of MT-induced PH and RV hypertrophy.

Mice treated with large amounts of MT exhibited severe infiltration of mononuclear cells and fibrosis in their lungs as well as pulmonary vasculitis with HO-1 induction. These histopathological changes seem to be similar to those described in a previous report (22). The MT-induced PH model has been used widely to analyze the effects and mechanisms of actions of various agents such as prostaglandin $I_2$ (3, 28). We believe that our murine MT model will be useful for examining the involvement of specific genes on PH and RV hypertrophy using transgenic animals.

Significant induction of HO-1 was observed in hypertrophied RV myocardium in our MT-treated mice. In hypertrophied myocardium, increased expression of proto-oncogenes such as c-fos and c-myc (2, 17) and increased synthesis and changes of myosin protein (26, 27) have been reported. These changes are thought to be adaptive responses to enhance the metabolic efficiency of contraction against increased pressure overload (27). At present, the pathophysiological significance of HO-1 induction in hypertrophied myocardium is unclear. However, stimulation of HO-1 expression in pressure-overloaded myocardium seems to be a general response, as HO-1 induction was also observed in a pulmonary artery banding model (15). Moreover, we recently detected HO-1 protein immunohistochemically in endomyocardial biopsy specimens of hypertrophied myocardium (K. Ishikawa, unpublished result).

We examined the effect of inhibition of HO activity on the MT-induced responses in lungs and heart (600 MT + Sn group). These mice exhibited significantly more severe inflammatory changes in their lungs and RV hypertrophy compared with the 600 MT group. These results suggest that HO-1 may ameliorate the inflammatory responses to MT in lungs. In fact, antiinflammatory properties of HO-1 have been reported in lipopolysaccharide-treated animals (10, 37) during the acute-phase rejection of murine heart transplants (6) and in a carotid artery balloon injury model (36). As the mechanism of MT-induced lung inflammation has not been elucidated, it is difficult to determine the mechanism(s) responsible for the antiinflammatory properties of HO in this model. These properties may be attributable to the reaction products, biliverdin/bilirubin and CO. Both biliverdin and bilirubin have been shown to inhibit lipid peroxidation and reduce the production of reactive oxygen species (29, 33). Indeed, such properties of these substances suppress endothelial and inflammatory cell interaction (9). Another reaction product, CO, has biological properties similar to nitric oxide in that it enhances soluble guanylyl cyclase activity. (23, 30). Vasodilatory action of CO on the pulmonary artery may reduce pulmonary arterial pressure and relieve the resultant RV hy-

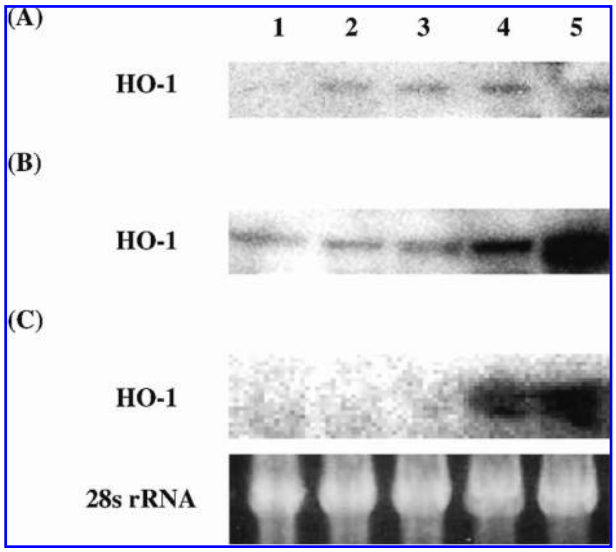


**FIG. 4. HO-1 expression in the lung and the RV after MT treatment.** Lane 1: control. Lane 2: 60 MT. Lane 3: 300 MT. Lane 4: 600 MT. Lane 5: 600MT + Sn. **(A** and **B)** Western blot analyses. Twenty micrograms of protein from lung **(A)** and RV **(B)** homogenates was subjected to 10% SDS-PAGE gel, blotted onto a PVDF membrane, incubated with anti-HO-1 antibody, and detected using the ECL chemiluminescent detection system. **(C)** Northern blot analysis. Fifteen micrograms of total RNA was electrophoresed in a formaldehyde/1% agarose gel, blotted with a nylon membrane, and hybridized with HO-1 probe as described in Materials and Methods. 28S rRNA was visualized by ethidium bromide staining.

pertrophy. Recently, Yet *et al.* reported that HO-1 knockout mice exhibited marked dilation and degeneration of RV after hypoxia-induced PH (40).

The prognosis of patients with pulmonary hypertension due to primary PH and chronic pulmonary thromboembolism is not satisfactory even after lung transplantation. Further studies will be necessary to understand the pathophysiology of PH and RV hypertrophy, and we hope this novel murine PH model will be used in future research. In addition, the function of HO-1 induction in hypertrophied myocardium should be elucidated.

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## ABBREVIATIONS

BW, body weight; CO, carbon monoxide; HO, heme oxygenase; LV, left ventricle; MT, monocrotaline; PAT, pulmonary artery thickness; PH, pulmonary hypertension; PVDF, polyvinylidene difluoride; RV, right ventricle; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SnPP IX, Sn-protoporhyrin IX.

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