Hemin Treatment Abrogates Monocrotaline-Induced Pulmonary Hypertension

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Abstract: Treatment of rats with monocrotaline (MCT), a pyrrolizidine alkaloid plant toxin, is known to cause pulmonary hypertension (PH), and it has been used as a useful experimental model of PH. Recent findings suggested that pulmonary inflammation may play a significant role in the pathogenesis of MCT-induced PH. We also demonstrated that, following MCT administration to rats, there was a significant and sustained increase in the pulmonary expression of heme oxygenase-1 (HO-1), which is known to be induced by various oxidative stresses, including inflammation and free heme, and is thought to be essential in the protection against oxidative tissue injuries. In this study, we administered hemin (ferriprotoporphyrin chloride, 30 μmol/kg b.w., subcutaneously), a potent inducer of HO-1, every 3 days to rats following subcutaneous administration of MCT (60 mg/kg) and examined its effect on MCT-induced PH and pulmonary inflammation. MCT administration caused pulmonary arterial wall thickening with marked elevation of right ventricular pressure, in association with prominent pulmonary inflammation as revealed by the increase in gene expression of tumor necrosis factor-α and the number of infiltrated neutrophils in the lung. In contrast, hemin treatment of MCT-administered animals, which led to a further increase in pulmonary HO-1 mRNA expression, significantly ameliorated MCT-induced PH as well as tissue inflammation. These findings suggest that hemin treatment ameliorates MCT-induced PH possibly mediated through induction of pulmonary HO-1 which leads to the attenuation of pulmonary inflammation.

Key Words: Heme oxygenase-1, hemin, inflammation. monocrotaline, pulmonary hypertension, tumor necrosis factor-α.

INTRODUCTION

Pulmonary hypertension (PH) is a serious and progressive disorder with poor prognosis [1, 2]. The pathophysiology of PH remains elusive, but recent findings suggest that oxidative stress due to pulmonary inflammation [3, 4] may be involved in its pathogenesis [5, 6]. Treatment of animals with monocrotaline (MCT), a pyrrolizidine alkaloid, causes significant pulmonary inflammation and cardiac hypertrophy, ultimately developing PH, and is used as an experimental model of PH [4]. Heme oxygenase-1 (HO-1), the rate-limiting enzyme in heme catabolism, is induced not only by its substrate heme but also by oxidative stress [7-10]. HO-1 has been considered to confer protection against tissue injuries due to various stimuli, including oxidative stress [7-10]. Our previous findings suggested that HO-1 may play a significant role in the protection of lung tissue injuries caused by MCT treatment [11]. With this point in mind, we examined in the present study the effect of administration of hemin, an oxidized form of heme that is available as a chemical, to MCT-treated rats. We report here that hemin treatment significantly ameliorated MCT-induced PH, together with the attenuation of pulmonary inflammation. Our findings also demonstrated that treatment of MCT animals with hemin further increased gene expression of HO-1 than that caused by MCT treatment alone. These findings suggest that hemin treatment ameliorates MCT-induced PH by induction of HO-1 which may contributes to the attenuation of pulmonary inflammation.

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RESULTS

Effect of Hemin Treatment on MCT-Induced PH and Lung Histology

We administered hemin to MCT-treated rats and examined its effect on RV pressure and lung morphology. At 3 weeks after MCT treatment, RV pressure in animals significantly increased compared with that in untreated control animals ($P<0.05$) (Fig. (**1**)) [11]. Its level was ~2.5 times greater than that of control animals. In contrast, hemin treatment of MCT animals resulted in a marked decrease in RV pressure ($P<0.05$, *vs*. Vehicle-treated MCT animals) that was essentially similar to that of untreated control animals (Fig. (**1**)). MCT-treated animals showed extensive infiltration of inflammatory cells in the lung interstitium and thickening of pulmonary arterial walls at 3 weeks after MCT treatment, while sections of the lung from control animals were essentially normal (Fig. (**2**)) [11]. Hemin-treated MCT animals showed markedly lesser infiltration of inflammatory cells and reduction of wall thickness of pulmonary arteries, compared with MCT treatment alone. These results indicate that hemin treatment significantly ameliorated MCT-induced PH as well as the infiltration of inflammatory cells.

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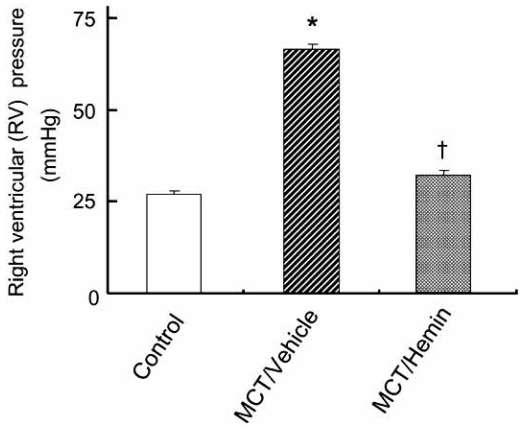

Fig. (1). Effect of hemin treatment on cardiac right ventricular (RV) pressure in monocrotaline (MCT)-induced pulmonary hypertension.

Hemin (30 μmol/kg) or vehicle was administered to rats subcutaneously every 3 days after MCT treatment (60 mg/kg, s.c.). Control rats received physiological saline. RV pressure was measured at 3 weeks after MCT treatment as described in "Materials and Methods". *Control, control animals; MCT/Vehicle, MCT with vehicle treatment; MCT/hemin, MCT with hemin treatment.* RV pressures are shown as mean ± SEM (N = 6 for each group). Statistical analysis by analysis of variance with Scheffé's *F*-test. **p* < 0.05 *vs* Control; †*p* < 0.05 *vs* MCT/Vehicle.

Effect of Hemin Treatment on Pulmonary Inflammation

Next, we examined the extent of pulmonary inflammation by assessing gene expression of tumor necrosis factor (TNF)-α and the number of infiltrating neutrophils into the lung. While pulmonary TNF-α mRNA was hardly detectable in untreated control rats, the level in MCT-treated animals was markedly increased at 2 weeks after the treatment (*P*<0.05, *vs*. untreated control animals) (Fig. (**3A**)) [11]. In contrast, the level in the hemin-treated MCT animals was markedly decreased (*P*<0.05, *vs*. Vehicle-treated MCT animals) and reached almost the same level as in the untreated control animals (Fig. (**3A**)). Consistent with changes in TNF-α gene expression, the number of infiltrating neutrophils markedly increased in MCT-treated animals compared with that in the untreated control animals (*P*<0.05) (Fig. (**4**)). Neutrophils were homogenously distributed in the lung interstitium of MCT-treated animals. In contrast, neutrophil recruitment was markedly reduced in the lung of hemin-treated MCT animals, and the number was significantly decreased (*P*<0.05, *vs*. Vehicle-treated MCT animals) (Fig. (**4**)). These findings thus indicate that hemin treatment attenuates MCT-induced inflammatory changes, including suppression of neutrophil recruitment.

Effect of Hemin Treatment on HO-1 Gene Expression

Since hemin is known as a potent inducer of HO-1 in various types of cells [12] including pulmonary cells [13], we examined the effect of hemin treatment on the expression of HO-1 mRNA in the lung. HO-1 mRNA was barely detectable in the lungs of untreated-treated control animals (Fig. (**3B**)). However, HO-1 mRNA level was significantly increased in the lungs of MCT-treated animals at 2 weeks after the treatment (*P*<0.05 *vs*. untreated control animals) (Fig. (**3B**)) [11]. Hemin administration to MCT-treated animals resulted in a further increase in HO-1 mRNA level at 2 weeks after MCT treatment (*P*<0.05, *vs*. Vehicle-treated MCT animals). The level of HO-1 mRNA in the lungs of hemin-treated MCT animals was approximately 5-fold greater than that of MCT-treated animals. These results indicate that hemin treatment significantly augmented the MCT-induced level of HO-1 mRNA.

DISCUSSION

The present study demonstrated that hemin treatment markedly ameliorated MCT-induced PH. Hemin treatment also significantly attenuated MCT-induced pulmonary inflammation. Our findings also showed that, while MCT treatment alone caused a substantial increase (~20-fold) in HO-1 mRNA, hemin treatment further increased its level by 5-fold. These results suggest that amelioration of MCT-induced PH by hemin treatment is possibly mediated by its potent ability to induce HO-1. Since certain synthetic heme analogues has been shown to possess the ability to simultaneously inhibit as well as induce the enzyme HO [14], the effect of hemin administration on HO activity in MCT-treated animals should be clarified in future study.

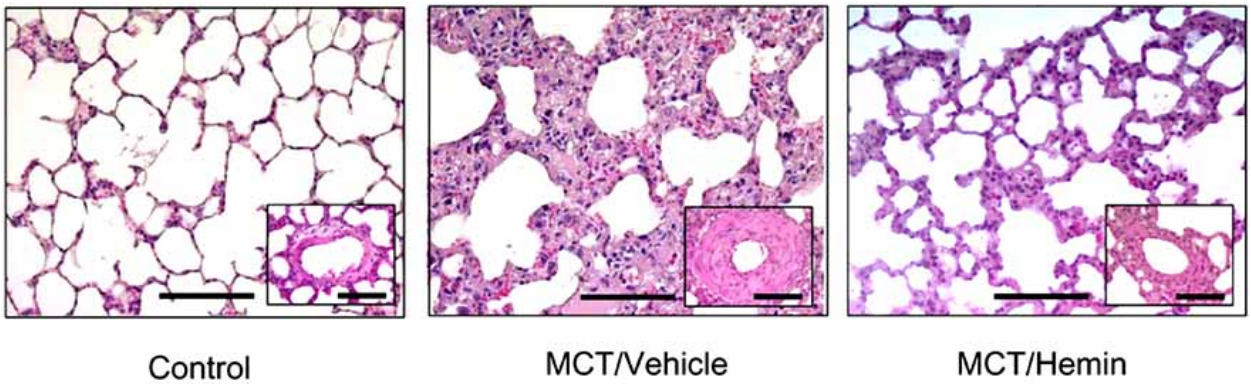

Fig. (2). Effect of hemin treatment on histological changes in the lung in monocrotaline (MCT)-induced pulmonary hypertension.

Hemin (30 μmol/kg) or vehicle was administered to rats subcutaneously every 3 days after MCT treatment (60 mg/kg, s.c.). Control rats received physiological saline. Three weeks after MCT treatment, lung sections were stained with hematoxylin and eosin. *Control, control animals; MCT/Vehicle, MCT with vehicle treatment; MCT/hemin, MCT with hemin treatment. Insets;* Pulmonary artery. Representative photomicrograph after each treatment from at least three independent experiments. The bar represents 100 μm.

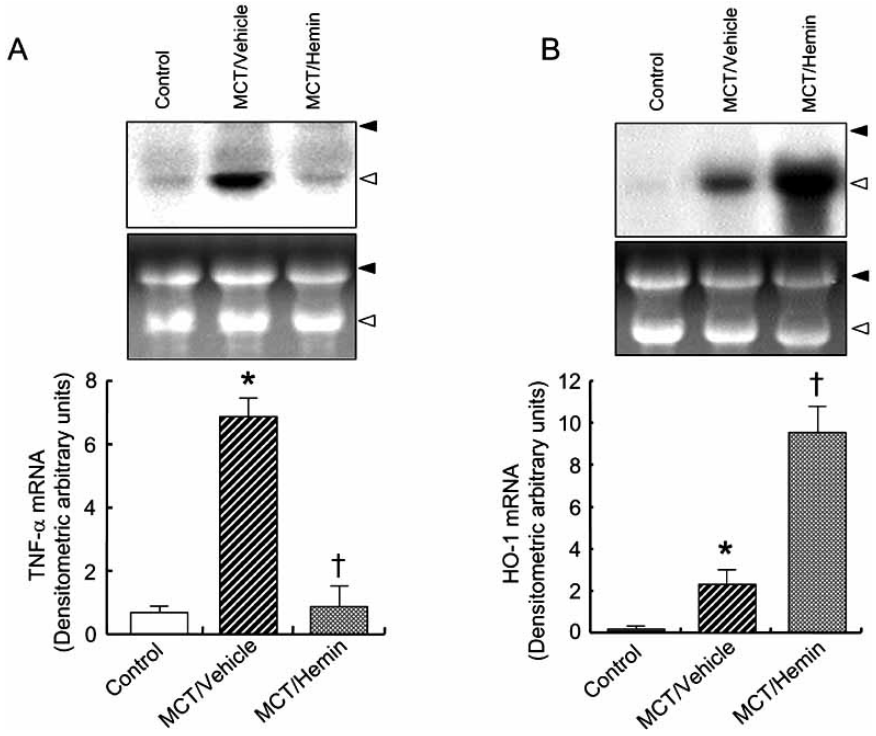

Fig. (3). Effect of hemin treatment on gene expression of tumor necrosis factor (TNF)-α and heme oxygenaes-1 (HO-1) in the lung in monocrotaline (MCT) -induced pulmonary hypertension.

Hemin (30 μmol/kg) or vehicle was administered to rats subcutaneously every 3 days after MCT treatment (60 mg/kg, s.c.). Control rats received physiological saline. Two weeks after MCT treatment, lungs were removed for Northern blot analysis as described in "Materials and Methods". *Control, control animals; MCT/Vehicle, MCT with vehicle treatment; MCT/hemin, MCT with hemin treatment. Top,* Shown are the autoradiographic signals of RNA blot hybridized with [α-^{32}P]dCTP-labeled TNF-α (A) and HO-1 (B) cDNA, respectively. Open and closed arrowheads next to the autoradiogram indicated the position of 18S and 28S ribosomal RNA corresponding to the ethidium bromide stained gels shown below, respectively. *Bottom,* The levels of TNF-α (A) and HO-1 (B) mRNA are expressed as densitometric arbitrary units. Data are expressed mean ± SEM (N = 3 for each group). Statistical analysis by analysis of variance with Scheffé's *F*-test. **p* < 0.05 *vs* Control; †*p* < 0.05 *vs* MCT/Vehicle.

MCT administration caused severe PH as confirmed by a marked increase in RV pressure. In contrast, hemin administration to MCT-treated animals markedly suppressed the MCT-mediated increase in RV pressure (Fig. (**1**)). In addition, lung sections of hemin-treated MCT animals showed a significant reduction of wall thickness of the pulmonary artery compared with that in MCT-treated animals (Fig. (**2**)). Thus, these findings clearly indicated that hemin treatment can abrogate the development of PH induced by MCT.

Our study also demonstrated that hemin administration to MCT-treated animals resulted in a significantly lower TNF-α mRNA level compared with that in hemin-untreated control animals (Fig. (**3A**)). Furthermore, histological examination revealed that hemin treatment markedly decreased the infiltration of inflammatory cells and reduced the recruitment of neutrophils to the lung in MCT animals (Figs. (**2**) & (**4**)), suggesting that hemin treatment attenuates MCT-induced PH by suppressing pulmonary inflammatory cell infiltration [4]. It has been reported that inhibition of inflammation by interleukin-1 receptor antagonists, or by an antibody that neutralizes monocyte chemotactic and activating factor/monocyte chemoattractant protein-1 alleviates MCT-induced pulmonary hypertension [15, 16]. Very recently, interleukin-10, anti-inflammatory cytokine, has also been reported to prevent the development of a rat model of MCT-induced PH [17]. Thus, in addition to the hemin-mediated protection, these mechanisms may constitute an additional mechanism in the protection against MCH-induced PH. It is unclear at present whether hemin treatment may involve these immunological modulations in its protective mechanism, but the question clearly remains as an important one for a future study.

The mechanism behind the anti-inflammatory effect of hemin may thus remain somewhat elusive. However, our results clearly demonstrated that administration of hemin to MCT-treated animals further increased the MCT-induced level of pulmonary HO-1 mRNA (Fig. (**3B**)). There are abundant evidence in various models that HO-1 has a potent anti-inflammatory and anti-oxidative property in various tissues and organs [7-10]. For instances, overexpression of human HO-1 has been shown to attenuate TNF-α-mediated inflammation injury in endothelial cells [18]. Moreover, very recently, it has been reported that hemin pretreatment induced a significant decrease in oxidative stress and TNF-α plasma levels with a significant increase of interleukin-10 plasma levels *via* HO-1 induction in lipopolysaccharide-treated rats [19]. Thus, hemin-mediated induction of HO-1 in the MCT-PH model constitutes another example of the po-

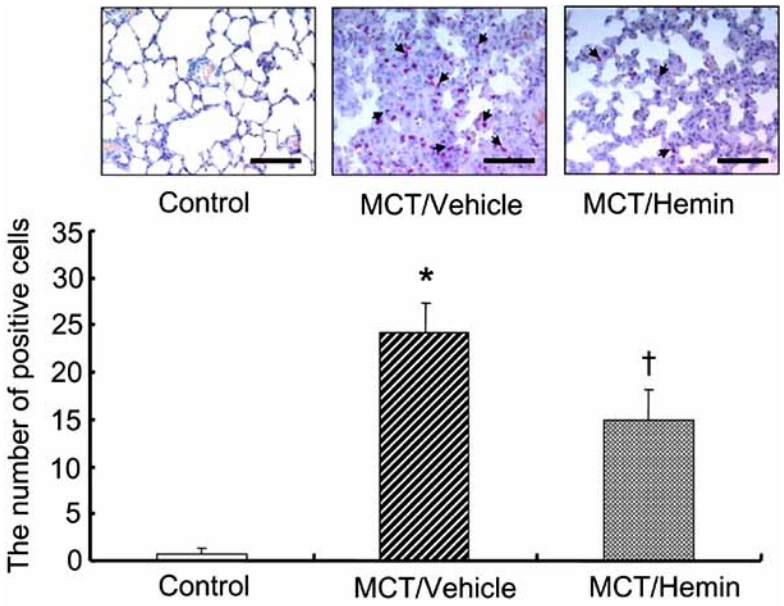

Fig. (4). Effect of hemin treatment on neutrophil infiltration into lung in monocrotaline (MCT)-induced pulmonary hypertension.

Hemin (30 μmol/kg) or vehicle was administered to rats subcutaneously every 3 days after MCT treatment (60 mg/kg, s.c.). Control rats received physiological saline. Three weeks after MCT treatment, lung sections were subjected to Naphthol AS-D chroloacetate esterase staining. *Control, control animals; MCT/Vehicle, MCT with vehicle treatment; MCT/hemin, MCT with hemin treatment. Top;* Representative photomicrograph of six independent experiments. Arrows indicate the positively stained cells. The bar represents 100 μm. *Bottom;* The number of neutrophils in five non-consecutive sections per rat at a magnification of x400. Data are expressed as mean ± SEM (N=6 for each group). Statistical analysis by analysis of variance with Scheffé's *F*-test. **p* < 0.05 *vs* Control; †*p* < 0.05 *vs* MCT/Vehicle.

tent anti-inflammatory and anti-oxidative property of HO-1 induction in the lung. In line with this conclusion, it has been reported that inhibition of HO activity by tin-protoporphyrin potentiated inflammatory changes in the lung and resultant RV hypertrophy in the MCT-induced PH model [20]. Very recently, rapamycin, an immunosuppressive agent, has been shown to ameliorate MCT-induced PH *via* HO-1 induction in rats [21]. In addition, a low dose of inhalation of CO, which is a physiological product of the HO reaction, reversed the established MCT-induced PH [22].

In summary, our study demonstrated that hemin treatment after MCT administration abrogated the development of PH induced by MCT, which is at least in part mediated by its potent induction of HO-1. Our findings also indicate that HO-1 induced by hemin treatment might serve as an important protective measure, and hemin may be a novel target for modulating the development of PH, although further studies to elucidate the dose-dependent effect of hemin on PH and to analyze the mechanism(s) behind the anti-inflammatory effect of hemin are clearly needed.

EXPERIMENTAL SECTION

Animals and Treatments

The studies reported herein conform to guidelines for the care and use of laboratory animals established by Animal Use and Care Committee of the Okayama University Medical School. Male Sprague-Dawley rats weighing 150-200 g were purchased from Charles River (Yokohama, Japan). They were housed in a temperature-controlled (25°C) room with alternating 12 h/12 h light/dark cycles and were allowed free access to water and chow diet until the start of experiments.

PH was induced by MCT treatment as described previously [11]. Animals were injected subcutaneously with MCT (Sigma Chemical Co., St. Louis, MO; 60 mg/kg body weight (b.w.)) which was dissolved in 0.1N HCl and then pH was adjusted to 7.4 with 0.1N NaOH. The final concentration was a 2% MCT solution (w/v). Control rats received the same volume of physiological saline. After the injection, animals were returned to cages and allowed free access to food and water. MCT animals were divided into the following two groups: treatment with hemin (ferriprotoporphyrin chloride, 30 μmol/kg b.w., subcutaneously) every 3 days after MCT treatment and treatment with the same volume of physiological saline every 3 days after MCT treatment. Hemin was dissolved in an alkaline solution (0.1N NaOH), and then reconstituted in physiological saline [23]. We previously reported that treatment of hemin with this dose significantly increased hepatic HO activity in rats without any adverse effect [23]. Under light anesthesia with ethyl ether, animals were sacrificed by decapitation at each defined time point (0 to 3 weeks). Lungs were excised and frozen immediately in liquid nitrogen and stored at -80°C until use for the preparation of RNA.

Measurement of RV Pressure

RV pressure was measured as described previously [11]. Briefly, animals were anesthetized with intraperitoneal injection of sodium pentobarbital (20 mg/kg b.w.), intubated with a 16 G tube by tracheostomy, and ventilated with a rodent ventilator (SN-480-7^{TM}, Shinao Manufacturing Co. Ltd., Tokyo, Japan). An incision was made in the right side of the animal's neck, and dissection was performed to expose the right jugular vein. A curve tipped 22 G catheter (Arrow International Inc., Reading, PA) was inserted into the vein and

advanced into the RV. The catheter was connected to the pressure transducer linked to a polygraph system (RM-6000™, Nihon Kohden Corp. Tokyo, Japan), and RV systolic pressure was measured.

RNA Isolation and Northern Blot Analysis

Total RNA was isolated from rat tissues using Tri-Reagent™ (Sigma Chemical Co.) according to the manufacturer's protocol. Northern blotting was performed as described previously [11, 24]. Twenty micrograms of total RNA were subjected to electrophoresis in a 1.2% (w/v) agarose gel containing 6.5% (v/v) formaldehyde. After blotting on to a sheet of BIO-RAD Zeta-Probe™ membrane (Bio-Rad Laboratories, Richmond, CA), RNA samples were hybridized with [α-^{32}P] dCTP labeled cDNA probes for HO-1 [25] and TNF-α [26, 27], respectively, followed by washing under stringent conditions. The membrane was exposed to a sheet of Fuji Medical radiograph film with an intensifying screen at -70°C, and autoradiographs and 18S ribosomal RNA were quantified by using an image scanner (GelPrint™ 2000i, Genomic Solutions, Ann Arbor, MI) and a computerized image analysis software (Basic Quantifier™ version 3.0, Genomic Solutions). Relative amounts of radiolabelled cDNA that hybridized to the blots were normalized to 18S ribosomal RNA levels for loading errors.

Histological Study

For histological examination, lung tissue was fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 4-6 μm thickness. After deparaffinization and dehydration, sections were stained with hematoxylin and eosin for microscopic examination. The neutrophils were stained with the use of a naphthol AS-D chloroacetate esterase-staining kit (Sigma Chemical Co.). The number of positively stained cells was counted in five non-consecutive sections per rat at a magnification of x400.

Statistical Analysis

Statistical evaluation was performed with analysis of variance followed by Scheffé's *F*-test by using Statview software (Abacus Concepts, Berkeley, CA). Differences were considered as significant at $p < 0.05$. Data are presented as means ± SEM.

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ABBREVIATIONS

HO-1 = Heme oxygenase-1

MCT = Monocrotaline

PH = Pulmonary hypertension

TNF = Tumor necrosis factor

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