Heme Arginate Pretreatment Attenuates Pulmonary NF-κB and AP-1 Activation Induced by Hemorrhagic Shock *via* Heme Oxygenase-1 Induction

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Abstract: Hemorrhagic shock followed by resuscitation (HSR) induces oxidative stress that leads to acute lung injury. Heme oxygenase-1 (HO-1), the rate-limiting enzyme in heme catabolism, is induced by oxidative stress and is thought to play an important role in the protection from oxidative tissue injuries. We previously demonstrated that HO-1 induction by heme arginate (HA), a strong inducer of HO-1, ameliorated HSR-induced lung injury and inflammation. Cellular redox state is known to modulate the DNA biding activity of the transcription factors; nuclear factor-κB (NF-κB) and activator protein-1 (AP-1). In the present study, we treated rats with HA (30 mg/kg of hemin) 18 h prior to HSR and examined its effect on the DNA binding activity of NF-κB and AP-1 at 1.5 h after HSR. HSR significantly increased the DNA binding activity of NF-κB as well as AP-1, while HA pretreatment markedly attenuated the activities of these transcription factors. In contrast, administration of tin mesoporphyrin, a specific competitive inhibitor of HO activity, to HA-pretreated animals abolished the suppressive effect of HA on the activities of NF-κB and AP-1, and increased these activities to almost the same level as those in HSR animals. Our findings indicate that HA pretreatment can significantly suppress the increased activity of NF-κB and AP-1 induced by HSR by virtue of its ability to induce HO-1. Our findings also suggest that HO-1 induced by HA pretreatment ameliorates HSR-induced lung injury at least in part mediated through the suppression of the activities of these transcription factors.

Key Words*:* Activator protein-1, acute lung injury, heme arginate, heme oxygenase-1, hemorrhagic shock, inflammation, nuclear factor–κB, oxidative stress.

INTRODUCTION

Hemorrhagic shock followed by resuscitation (HSR) induces a systemic inflammatory response that results in acute lung injury (ALI) [1]. Although the pathophysiology of HSR-mediated ALI remains elusive, oxidative stress has been implicated as an important cause in its pathogenesis [2]. Heme oxygenase-1 (HO-1), the rate-limiting enzyme in heme catabolism [3], can be induced not only by its substrate heme [3] but also by oxidative stress [4], and is thought to confer protection against oxidative tissue injuries [4]. Heme arginate (HA), which is a water-soluble and stable reaction product of hemin with L-arginine [5], is known to strongly induce HO activity [6], and has been used for treatment of acute relapses of patients with acute hepatic porphyrias [7, 8]. HA treatment supplements heme for heme deficiency, and is thought to suppress the de-repression of non-specific δ-aminolevulinate synthase in the liver that occurs in acute relapses of these patients. We previously found that HO-1 induction by HA pretreatment ameliorated HSR-induced lung injury and inflammation [9]. However, the precise mechanism by which HO-1 confers protection against HSR-induced lung injury remains unclear. It is well known that oxidative stress modulates the activities of certain transcription factors, such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) [10-13]. The present study therefore examined the effect of HSR on the DNA binding activities of these transcription factors in the lung and their modulation by HA. We report here that HSR increased not only the activity of NF-κB but also that of AP-1 in the lung. HA pretreatment decreased the activities of both transcription factors induced by HSR. In contrast, administration of tin mesoporphyrin (SnMP), a specific competitive inhibitor of HO activity [14], abolished the suppressive effect of HA pretreatment. These findings indicate that HA pretreatment can significantly reduce the activities of NF-κB and AP-1 induced by HSR by virtue of its ability to induce HO-1. Our findings also suggest that HO-1 induced by HA pretreatment ameliorates HSR-induced lung injury at least in part mediated through the suppression of the activities of these transcription factors.

METHODS

Animal Treatments

Animal experiments were approved by the Animal Care Committee of Okayama University Medical School; care and handling of the animals were in accordance with National

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Institutes of Health guidelines. Male Sprague-Dawley rats weighing 350-400 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. Rats were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg) and then subjected hemorrhagic shock followed by resuscitation (HSR) as we described previously [9]. The sham-operated animals underwent all experimentation procedures except bleeding. HSR rats were randomly assigned to the following four groups: control with sham operation (Sham group; n=3), pretreatment with HA before HSR (HA/HSR group; n=3), pretreatment with vehicle before HSR (Vehicle/HSR group; n=3), pretreatment with HA followed by administration of SnMP before HSR treatment (HA/SnMP/HSR group; n=3). HA and SnMP solutions were prepared as described previously [9]. Fifteen point three mmol (2.67g) of HA (30 mg of hemin/kg) or the vehicle (1,2-propanediol (40%) and ethanol (10%) solution) was injected at 18 h before HSR, and SnMP (0.5 μmol/kg) was injected at 2 h before HSR *via* tail vein, respectively. We previously reported that treatment with 30 mg/kg of hemin in HA solution markedly induced functional HO-1 protein in the lung without any adverse effect, while administration of 0.5 μmol/kg of SnMP completely inhibited HA-induced HO-1 activity in the lung [9]. Thus, we chose these dosage of HA and SnMP in this study. Under light anesthesia with ethyl ether, animals were sacrificed by decapitation at 1.5 h after HSR, and lungs were frozen immediately in liquid nitrogen and stored at -80° until use for the preparation of nuclear extracts

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared from frozen liver sections as we described previously [16]. EMSA was also performed as we described previously [16]. Autoradiographs were quantified using an image scanner (GelPrint® 2000i, Genomic Solutions Inc., Ann Arbor, MI) and a computerized image analysis software (Basic Quantifier® Ver. 3.0, Genomic Solutions Inc.) [15, 25].

Statistical Analysis

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

RESULTS

The DNA binding activity of both NF-κB and AP-1 was detectable in the lung of sham-operated animals (Sham group) (Figs. **(1)** and **(2)**). NF-κB activity increased significantly in Vehicle/HSR animals (Fig. **(1)**). In contrast, its level in HA/HSR animals markedly decreased to ~10% of the level of Vehicle/HSR animals (Fig. **(1)**). Similar to changes in the DNA binding activity of NF-κB, the activity of AP-1 also significantly increased in Vehicle/HSR animals compared with that of sham-operated animals, and its level in HA/HSR animals markedly decreased to ~15% of the level of Vehicle/HSR animals (Fig. **(2)**). Administration of SnMP, a specific competitive inhibitor of HO activity, to HA-pretreated animals prior to HSR (HA/SnMP/HSR group) increased the levels of these transcription factors to almost the same level as observed in Vehicle/HSR animals (Figs. **(1)** and **(2)**). Consistent with our previous reports [15, 16], competition studies showed that the binding of the probe to NF-κB (Fig. **(1)**) and AP-1 (Fig. **(2)**) binding sequence was specific for each probe, as it was completely abolished by the authentic probe, but was not influenced by a non-specific probe.

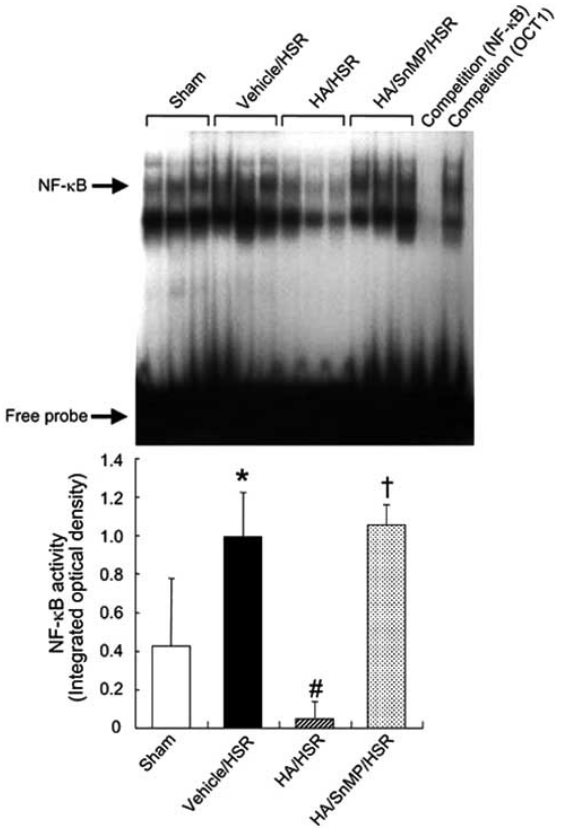

Fig. (1). Effect of HA pretreatment on the activation of NF-κB binding after HSR.

HA (equivalent to 30 mg of hemin/kg) or vehicle was administered to rats intravenously 18 h before HSR. SnMP (0.5 μmol/kg) was administered to rats intravenously 2 h before HSR. Lungs were excised 1.5 h after HSR for EMSA as described in "Materials and Methods". Autoradiographs (*top*) were quantified by image analyzer, and the levels of NF-κB activity are expressed as integrated optical density (*bottom*). *Sham,* sham-operated control; *Vehicle/HSR,* HSR with vehicle pretreatment; *HA/HSR;* HSR with HA pretreatment; *HA/SnMP/HSR,* HSR with HA pretreatment followed by SnMP administration; *Competition (NF-κB),* Vehicle/HSR with the addition of 100-fold excess of unlabeled NF-κB oligonucleotide; *Competition (OCT1),* Vehicle/HSR with the addition of 100-fold excess of unlabeled OCT1 oligonucleotide. Data are presented as means ± S.D. (n = 3 for each group). *$p <$ 0.05 *vs*. Sham; †$p < 0.05$ *vs*. Vehicle/HSR; #$p < 0.05$ *vs*. HA/HSR.

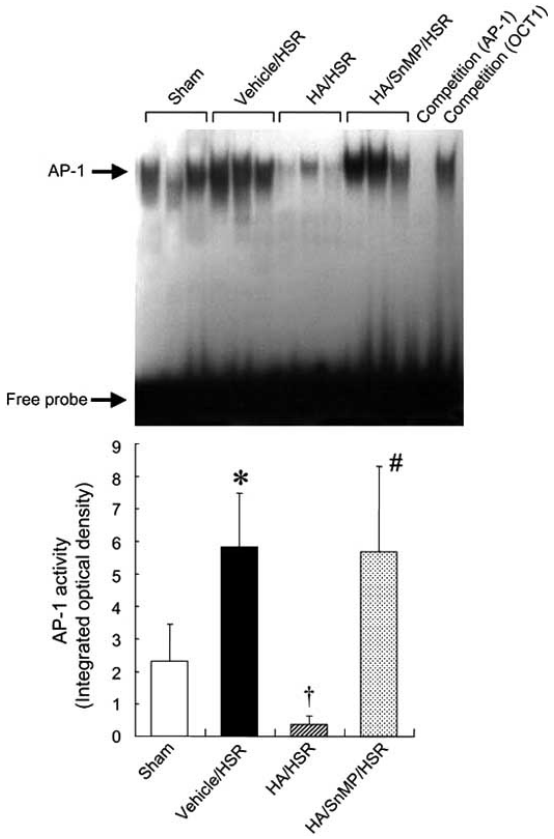

Fig. (2). Effect of HA pretreatment on the activation of AP-1 binding after HSR.

HA (equivalent to 30 mg of hemin/kg) or vehicle was administered to rats intravenously 18 h before HSR. SnMP (0.5 μmol/kg) was administered to rats intravenously 2 h before HSR. Lungs were excised 1.5 h after HSR for EMSA as described in "Materials and Methods". Autoradiographs (*top*) were quantified by image analyzer, and the levels of AP-1 activity are expressed as integrated optical density (bottom). *Sham,* sham-operated control; *Vehicle/HSR,* HSR with vehicle pretreatment; *HA/HSR;* HSR with HA pretreatment; *HA/SnMP/HSR,* HSR with HA pretreatment followed by SnMP administration; *Competition (AP-1),* Vehicle/HSR with the addition of 100-fold excess of unlabeled AP-1 oligonucleotide; *Competition (OCT1),* Vehicle/HSR with the addition of 100-fold excess of unlabeled OCT1 oligonucleotide. Data are presented as means ± S.D. (n = 3 for each group). **p* < 0.05 *vs*. Sham; †*p* < 0.05 *vs*. Vehicle/HSR; #*p* < 0.05 *vs*. HA/HSR.

DISCUSSION

Our results in this study showed that HSR increased the DNA binding activity of NF-κB and AP-1 in the lung, while HA pretreatment markedly attenuated the HSR-induced activities of these transcription factors. We also demonstrated that inhibition of HO activity by a specific HO inhibitor, SnMP, abolished the suppressive effect of HA pretreatment. Using the same model, we previously showed that HO-1 induction by HA pretreatment ameliorated the HSR-induced lung injury [9]. Thus, our findings also suggest that HO-1 induction by HA pretreatment ameliorates the lung injury induced by HSR at least in part by suppressing induced NF-κB and AP-1 activities by HSR.

Following HSR, the DNA binding activity of NF-κB significantly increased compared with that of sham-operated control animals (Fig. **(1)**) [17]. The activity of AP-1 also showed a significantly higher level than that of sham-operated control (Fig. **(2)**). It is known that reactive oxygen species (ROS) generated by ischemia-reperfusion plays an important role in the pathogenesis of the tissue injury after HSR [2]. It is also well known that oxidative stress increases the DNA binding activity of NF-κB in the cell [10, 11]. It has also been reported that ROS-dependent activation of hepatic AP-1 occurs in rats after HSR [18]. Thus, oxidative stress elicited by HSR in part may contribute to the increase in NF-κB activity and AP-1 activity after HSR.

HA pretreatment markedly down-regulated both HSR-induced NF-κB and AP-1 activities compared with those which were treated with HSR only (Figs. **(1)** and **(2)**). In contrast, the administration of SnMP, a specific competitive inhibitor of HO activity [14], to HA-pretreated animals abolished the suppressive effect of HA pretreatment on HSR-induced NF-κB and AP-1 activities, and increased their levels to almost the same level as those in HSR-treated animals (Figs. **(1)** and **(2)**). We previously reported that HA treatment markedly increased functional HO-1 protein in the lung [9]. We also confirmed that SnMP administration to HA-treated animals effectively inhibited the HA-induced HO activity [9]. Collectively, our findings indicate that HA pretreatment attenuated the increase of HSR-induced NF-κB and AP-1 activities *via* its induction of HO-1.

It should be noted that HO-1 oxidatively cleaves heme, and reduces the amount of this potent pro-oxidant. In addition, the HO reaction on heme yields carbon monoxide (CO), iron, and biliverdin IXα, which is then reduced to bilirubin IXα by biliverdin IXα reductase [3]. Both biliverdin IXα and bilirubin IXα function as potent endogenous anti-oxidants [19]. Although it is unclear to what extent each mechanism may participate, they would reduce the oxidative stress, and contribute to the decrease in the activities of NF-κB and AP-1 by HO-1. Activations of promoters of pro-inflammatory genes, such as tumor necrosis factor-α (TNF-α) and inducible nitric oxide synthase (iNOS) are known to be mediated in part by the activation of transcription factor NF-κB [20, 21]. It has also been reported that the DNA binding of AP-1 plays an important role in the transcription of TNF-α gene [22, 23]. Thus, the decrease of these transcription factors by HO-1 may also result in the suppression of pro-inflammatory response, leading to the amelioration of HSR-induced lung injury. In support of our hypothesis, we found that HO-1 induction by HA pretreatment down-regulated the gene expression of TNF-α and iNOS in the lung after HSR [9]. We have also demonstrated that HO-1 induction by glutamine ameliorates LPS-induced oxidative tissue injury in the intestine by attenuating the expression of TNF-α expression [24].

In summary, the present study demonstrated that HA pretreatment can significantly suppress the increased activity

of NF-κB and AP-1 induced by HSR by virtue of its ability to induce HO-1. Our results also suggest that HO-1 induction by HA pretreatment ameliorates HSR-induced lung injury at least in part mediated through the attenuation of the DNA biding activities of NF-κB and AP-1.

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ABBREVIATIONS

ALI = Acute lung injury

AP-1 = Activator protein-1

EMSA = Electrophoretic mobility shift assay

HA = Heme arginate

HO-1 = Heme oxygenase-1

HSR = Hemorrhagic shock followed by resuscitation

iNOS = Inducible nitric oxide synthase

LPS = Lipopolysaccharide

NF-κB = Nuclear factor-κB

ROS = Reactive oxygen species

SnMP = Tin mesoporphyrin

TNF-α = Tumor necrosis factor-α

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