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Is Regulation of Heme Catabolism Associated with Inflammation? Suggestions from Analysis of *cis*-Acting Elements in the Heme Oxygenase-1 Gene

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ヘム分解酵素ヘムオキシゲナーゼ-1遺伝子上流シスエレメントの解析

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Abstract

Cellular heme catabolism is essential for multiple physiologic processes such as iron recycling, antioxidant defense, control of inflammation, and primarily depends on the activity of heme oxygenase-1 (HO-1). Although induction of HO-1 by various stimuli has been known, the underlying mechanism is not clear. In this study, we performed *in silico* search for *cis*-acting elements present within 5' flanking region of HO-1 gene. In addition to known heme- and heat-operated elements, the analysis highlighted binding sites for inflammation-related transcription factors including NF-κB, PPARγ, and EOMES. These sites were specifically present in mammalian and avian HO-1 genes and the most frequently found in human sequence. Collectively, the analysis indicates that HO-1 gene is under the control of multiple signaling nodes, illustrating how HO-1 activity is optimally tuned in response to various physical and inflammatory stimuli

keywords：heme oxygenase-1, inflammation, heat shock, stress response

Introduction

Heme oxygenase-1 (HO-1) is a membrane-anchored protein and catalyzes oxidative degradation of heme to biliverdin IXa, carbon monoxide, and iron. Serving as a rate-limiting enzyme in heme catabolism, HO-1 plays a crucial roles in detoxification of potentially harmful heme, iron recycling from senescent erythrocytes, antioxidative defense, and production of carbon monoxide which is a potent signaling molecule in stress response[1)]. In the first clinical case of HO-1 deficiency, mutations in HO-1 gene (***hmox1***) are associated with anemia, elevated heme level in serum and macrophage, low serum bilirubin concentration, and chronic inflammation[2)]. Similarly, *hmox1*$^{-/-}$ mice presented iron deficit in serum, oxidative tissue injury, and abnormal sensitivity to inflammation[3)]. In endotherial cells and macrophage, inhibition of HO-1 sensitized

cells against oxidative insults and affected anti-inflammatory processes[4,5], illustrating the essentiality of HO-1 in stress and immune responses. This property in part depends on the ability of *hmox1* to respond to various stressors and inflammatory factors by drastically modulating its transcription level. Previous studies have identified many HO-1 inducers including heme, UV exposure, heat shock, transition metals, oxidants such as hydrogen peroxide, endotoxin, and inflammatory cytokines[6,7]. Such broad spectrum of transcriptional inducers, ranging from physical and chemical stimuli to biologically potent substances, is unique to *hmox1* gene, imparting fundamental cytoprotection required in various stressful conditions.

A number of studies sought to decipher the mechanism underlying induction of *hmox1* gene. First, HO-1 has been known to be one of heat shock proteins (HSPs), a set of proteins specifically induced upon elevation of temperature. Induction of HSPs are mainly controlled by heat shock factor 1 (HSF1), a transcription factor (TF) transmitting signals of thermal and proteostatic stresses[8]. At a normal temperature, HSF1 exists in the cytosol in a form of inactive protein complex. Upon elevation of temperature, HSF1 is released from an inactive complex to form a hyperphosphorylated trimer, translocates into nucleus, and binds to specific regions of genomic DNA called heat shock elements (HSEs); each protomer of trimerized HSF1 recognizes the sequence 5'-nGAAn-3' in an alternating orientation and, collectively, the consensus sequence of HSE is defined to be 5'-GAAnnTTCnnGAA-3'[9]. Indeed, multiple HSEs are present in the promoter region of *hmox1*. Similar to heat shock, heme induces HO-1 via specific TFs, namely, Nrf2 and Bach1. Both TFs belong to Cap'n'collar (CNC) family, form heterodimers with small Mafs (sMafs), and bind to the same sequence pattern; Nrf2::sMaf and Bach1::sMaf bind to Maf recognition elements (MAREs; 5'-TGACTCAGCA-3')[10]. Nrf2 has a transactivator domain hence a positive regulator of *hmox1* gene. Bach1 lacks activating moiety and serves as a repressor which counteracts Nrf2::sMaf[11]. Heme impedes Bach1-MARE interaction, resulting in a drastic enhancement of *hmox1* transcription[12]. Regarding other HO-1 inducers, especially those associated with inflammation, the mechanisms of HO-1 induction are not completely understood. HO-1 is inducible by treatment with inflammatory cytokines including IFN-γ[13]. We previously reported that *in vivo* LPS administration elevated serum IL-6 level and markedly induced HO1 expression in liver, kidney and lung[14]. Two copies of IL-6 responsive elements are present in the proximal promoter region of human *hmox1* gene[15].

In this study, we performed a sequence analysis of *hmox1* promotor to search for unique *cis*-acting elements. Our search focused on binding sites for inflammation-associated TFs including NF-κB, PPARγ, and EOMES. Sequence comparison among different species highlighted higher frequency of these TF sites in mammalian sequences compared to other vertebrates, pointing to the link between immune response and *hmox1* activation uniquely evolved within mammals.

Materials and Methods

ChIP-seq database search. To collect information about transcription factors (TFs) that interact with the upstream region of human *hmox1* gene, data mining through published ChIP-seq data sets was performed on ChIPBase[16]. 5 kbp upstream region of human *hmox1* was scanned for associations of various TFs. Upstream regions of *hmox2* and *hspa1a* genes were similarly scanned.

Sequence analysis. The sequences of 5 kbp upstream regions of *hmox1* genes were retrieved at Ensemble and NCBI genome browsers. Analyzed were genomic sequences of human (*Homo sapiens*), macaque (*Macaca mulatta*), cow (*Bos taurus*), rat (*Rattus norvegicus*), mouse (*Mus musculus*), chicken (*Gallus gallus*), lizard (*Anolis carolinensis*), frog (*Xenopus tropicalis*), zebrafish (*Danio rerio*). Putative bindings sites for 5 TFs were analyzed on GENETYX ver. 13 (GENETYX, Japan) based on the following consensus sequence patterns; 5'-TGACTCAGCA-3' (MARE)[17], 5'-GAAnnTTCnnGAA-3' (HSE)[18], 5'-GGP$_u$nTTTCC-3' (NF-κB/RelA::p50)[19], 5'-AGGTCAnAGGTCA-3' (PPARγ::RXR)[20], 5'-AGGTGP$_y$GA-3' (EOMES)[19], where critical bases are underlined. Replacement of one noncritical base was tolerated in the search.

Results and Discussion

Unique spectrum of TFs bound to the upstream region of hmox1 gene. For genome-wide analyses of TF binding sites, ChIP-seq experiments have been extensively utilized. The accumulating data sets are available at open-access databases including NCBI GEO, ENCODE, and modENCODE. These data sets were integrated by Yang and colleagues, providing a comprehensive information of more than 8 million TF binding sites (ChIPBase)[16]. Using this database, we searched for TFs interacting with the 5 kbp upstream region of human *hmox1* gene. Total 107 TFs were found (Fig. 1). For comparison, *hmox2*, the gene encoding constitutively expressed form of heme oxygenase, was similarly analyzed, indicating 47 TFs which is significantly less than *hmox1*. Reflecting the unique ability of *hmox1* gene to sense heme, interaction with Nrf2 and Bach1 was specifically observed for *hmox1*. Also, as expected, HSF1 association was found for *hmox1* as well as *hspa1a*, a gene encoding a representative heat shock protein (HSP70). In total 73 TFs were exclusively found for *hmox1*. Representative TFs are shown in Fig. 1.

Validation of MARE and HSE in the upstream region of HO-1 gene. Heme is a robust inducer of HO-1 and its transmission depends on MARE-targeted TFs (Nrf2::sMaf and Bach1::sMaf). As described previously[21], three MAREs are present in the 5 kbp upstream region of human *hmox1* gene (Fig. 2). In all examined vertebrates from fish to human, up to 4 MAREs were found within 5 kbp upstream region of *hmox1*, suggesting the possibility that heme-dependent induction of *hmox1* gene is evolutionarily conserved among practically all vertebrates. It is of note that no MARE site was found after sequence randomization.

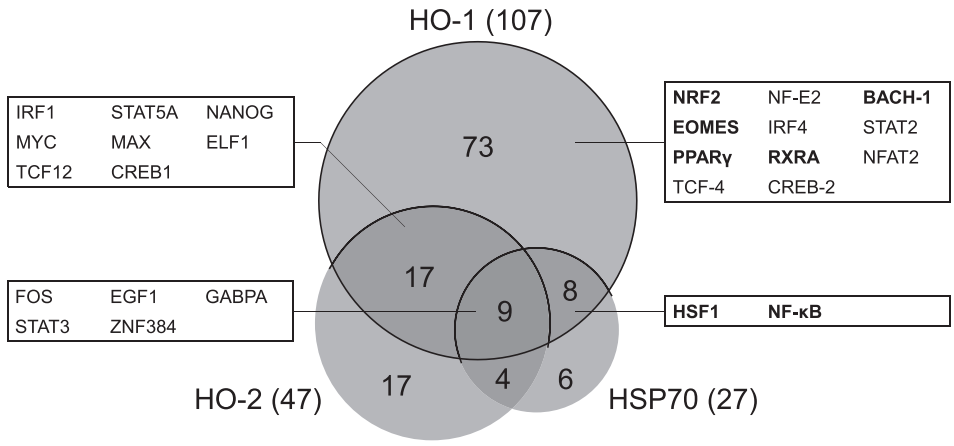

Fig.1 Transcription factors known to interact with 5' flanking regions of human HO-1, HO-2, and HSP70 genes. Interactions were based on integrated ChIP-seq data retrieved from NCBI GEO, ENCODE, and modENCODE. Number of transcription factors (TFs) in each intersection is represented. The total number of TFs for each gene is indicated in parentheses. TFs subjected to *cis*-element analyses in Fig.2 and 3 are highlighted in *bold*.

Heat shock is another well-documented stimulus for HO-1 induction although the degree of the response varies among different species[21]. Two HSEs are present in the 5 kbp upstream region of human *hmox1*. Paradoxically, it has been demonstrated in many studies that in human cells HO-1 is only modestly upregulated by heat shock[22]. In contrast, rat HO-1 is robustly induced upon heat shock[23]. It has been proposed in the previous study[7] that the strong response to heat shock in rat may be attributed to the multiplicity of HSE repeats within the region proximal to the transcription start site (around -500 bp position; *dashed* square in Fig. 2). Unlike rat, sequences of other mammals have single HSEs in the proximal region. As shown in Fig. 2, there are additional HSEs in more distal region (1-5 kbp upstream) except for macaque sequence. These distal HSEs were implicated in the modest response to heat shock[7,24]. Interestingly, unlike MARE, HSE is absent from the sequences of nonmammal vertebrates (chicken, lizard, frog and fish; data not shown), highlighting HSE-mediated induction of HO-1 as a specialized stress response system evolved within Mammalia.

Binding sites for inflammation-related TFs in the upstream region of HO-1 gene. Organs of HO-1 knockout mice showed higher susceptibility to inflammation[3] and its repression exacerbated inflammatory injury in a septic animal model[6,25], establishing evidences for cytoprotective role of HO-1 in immune response (IR). Most IR-associated genes are under the control of common TFs such as NF-κB. Based on the list shown in Fig. 1, we sought to search for putative binding sites of well-known IR-related TFs, i.e., NF-κB, PPARγ::RXR, and EOMES. Among 9 species from vertebrates, the number of these IR-related TFs is the highest in human, least in chicken, and none in fish or randomized sequence. Seemingly, these *cis*-elements may not have randomly appeared within the sequence but strategically evolved.

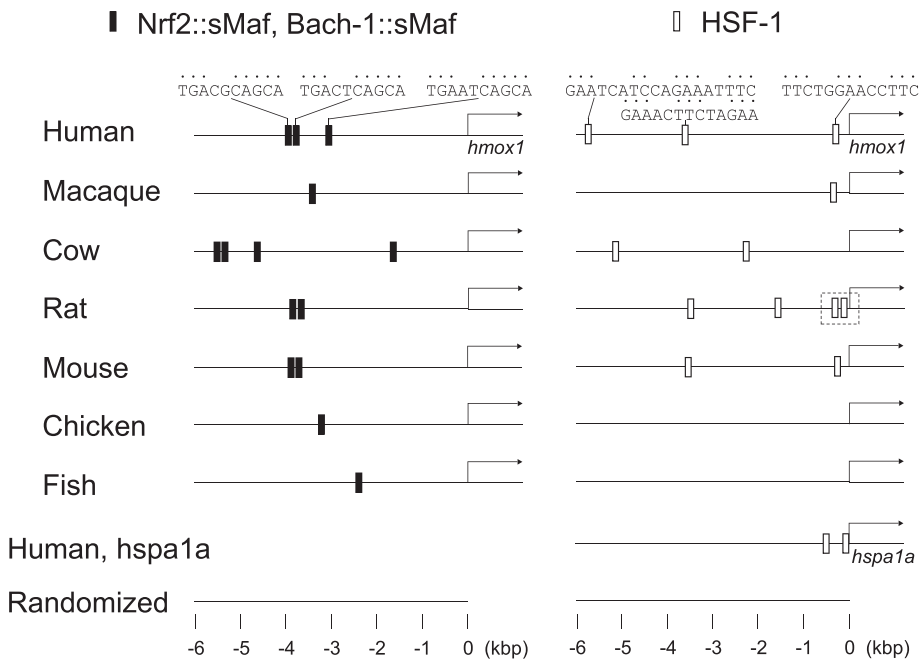

Fig.2 Diagrams of stress responsive *cis*-acting elements in 5' flanking regions of HO-1 genes of various species within vertebrata. Human sequences are also shown above the diagram. Critical bases are highlighted by *dots*. Dashed square represents a HSE repeat described in the main text. The gene of heat shock protein 70 (*hspa1a*) and randomized sequence are also included for comparison.

Importantly, NF-κB has been implicated in HO-1 induction; stimulants of NF-κB signaling pathway such as EGGC (epigallocatechin-3-gallate) upregulated HO-1 and, reciprocally, NF-κB inhibitor abolished HO-1 induction by curcumin or nordihydroguaiaretic acid[26]. NF-κB is formed by homo- or hetero-dimerization of Rel family proteins (RelA, RelB, c-Rel, p52, and p50). The target sequence of NF-κB significantly differs depending on the combination of Rel subunits. RelA::p50 heterodimer is the best characterized and in most cell types the most abundant. All possible target sequences of NF-κB were searched within the upstream region of human *hmox1*. The analysis specifically identified the sites for RelA::p50, which agrees with the ChIP-seq profile[27]. In human sequence, RelA::p50 site is present at -2.2 kbp position (Fig. 3). This site is distinct from previously proposed site for another NF-κB variant which was found within a proximal region (between -480 bp to +110 bp)[28].

Heterodimer of PPARγ and retinoid X receptor (RXR) functions as a nuclear receptor that suppresses NF-κB signal and thereby plays anti-inflammatory role. Upon ligand binding, PPARγ::RXR docks at a PPAR responsive element (PPARE)[29] which is defined as 5'-AGGTCAnAGGTCA-3'. Similar to NF-κB binding sites, PPAREs are conserved among vertebrates except for fish. It is interesting that HO-1 gene has *cis*-elements for both counteracting transactivators, i.e., pro-inflammatory and anti-inflammatory factors. Coexistence of PPARγ::RXR and NF-κB binding sites for one gene is not unusual. For instance, TNIP1 is

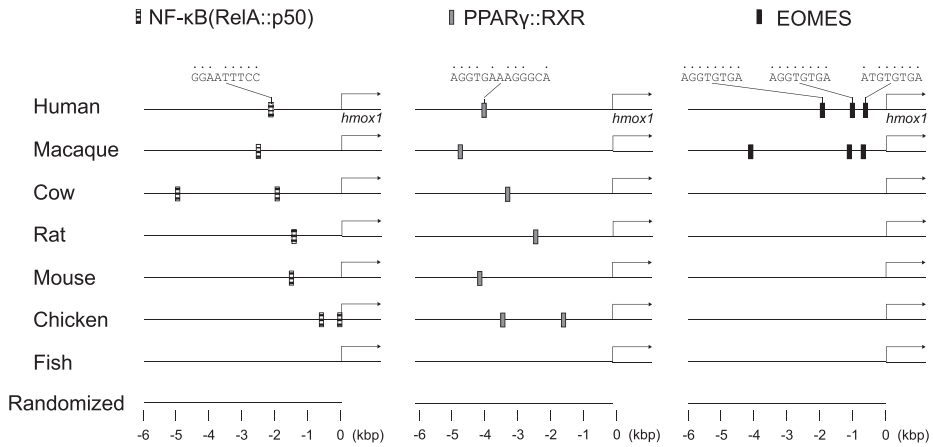

Fig.3 Diagrams of inflammation-associated *cis*-acting elements in 5' flanking regions of HO-1 genes of various species within vertebrata. Diagrams are represented as shown in Fig. 2.

additively induced by both TFs, allowing TNIP1 induction through various stages of immune response[30], which may be also the case with HO-1.

EOMES regulates subtype differentiation of helper T cell[31]. Depending on the balance of various cytokines, helper T cells convert to either Th1, Th2, and Th17 phenotype[32]. EOMES functions as a pivotal transcription repressor, suppressing a group of genes required for pro-inflammatory processes in Th17[31]. EOMES binding sites were found in primate sequences (human and macaque), presumably serve as HO-1 repressor in EOMES-positive lymphocytes. In EOMES-negative Th17 cells, HO-1 is expected to be released from EOMES-mediated repression and engaged in inflammation control and/or cytoprotection.

Conclusions

Sequence analysis highlighted the presence of various TF binding sites in the upstream region of *hmox1* gene. First, distributions of MARE and HSE, which have been known to operate heme- and heat shock-dependent induction of HO-1, were confirmed. The result demonstrated that, in vertebrates (human, macaque, cow, rat, mouse, chicken and fish), the upstream region of *hmox1* gene commonly contains MAREs, implicating conserved capability of vertebrate *hmox1* gene to respond to heme. In contrast, HSEs were specifically found for human, macaque, cow, rat and mouse *hmox1* genes and seemingly evolved within Mammalia. Second, the sequence analysis suggested that *hmox1* gene is regulated by inflammation-related TFs, i.e., NF-κB, PPARγ, and EOMES. Unlike MARE, binding sites for these pro- and anti-inflammatory TFs are specifically found for avian and mammalian *hmox1* and the most frequently found in human sequence. Collectively, HO-1 activity is flexibly calibrated by multiple TFs and cognate *cis*-elements within the upstream region of the gene, imparting optimal level of heme catabolism in distinct cell types in various conditions.

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