Biochimica et Biophysica Acta 1840 (2014) 2351–2360

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Hemopexin-dependent heme uptake via endocytosis regulates the Bach1 transcription repressor and heme oxygenase gene activation

Hiroshi Hada [a,b], Takuma Shiraki [c], Miki Watanabe-Matsui [a], Kazuhiko Igarashi [a,b,*]

[a] Department of Biochemistry, and Center for Regulatory Epigenome and Disease, Tohoku University Graduate School of Medicine, Sendai 980-8575, Japan
[b] CREST, Japan Science and Technology Agency, Sendai 980-8575, Japan
[c] Faculty of Biology-Oriented Science and Technology, Kinki University, Nishimitani, Kinokawashi, Wakayama 649-6493, Japan

ARTICLE INFO

Article history:
Received 5 November 2013
Received in revised form 24 February 2014
Accepted 27 February 2014
Available online 5 March 2014

Keywords:
Heme
Hemopexin
Bach1
Heme oxygenase-1
Oxidative stress
Endocytosis

ABSTRACT

Background: Intracellular heme plays versatile roles in a variety of physiological processes including mitochondrial respiration. Heme also induces the expression of genes such as heme oxygenase-1 (HO-1) by inactivating the transcription repressor Bach1 through direct binding. However, the source of heme for the regulation of the Bach1–HO-1 axis has been unclear. Considering that extracellular heme exists as a complex with hemopexin (Hx) in serum under the physiological conditions, heme–Hx complex may deliver heme for the gene regulation.
Methods: Using a mammalian expression system, high secretory recombinant Hx (rHx) was developed. We examined the effects of rHx-bound heme on HO-1 expression and Bach1 in Hepa-1c1c7 liver cells and THP-1 macrophage cells. We investigated the uptake pathway of rHx-bound heme by treating cells with chlorpromazine (CPZ).
Results: rHx-bound heme induced the expression of HO-1 and decreased the level of Bach1 protein. CPZ inhibited the induction of the HO-1 expression by rHx-bound heme.
Conclusion: rHx-bound heme was internalized into the cells via endocytosis, resulting in HO-1 expression and inactivation of Bach1.
General significance: The Bach1-dependent repression of the HO-1 expression is under the control of the Hx-dependent uptake of extracellular heme. Heme may regulate Bach1 as an extracellular signaling molecule.

1. Introduction

The transcription repressor Bach1 plays a critical role in constraining the expression of oxidative stress response genes [1]. For example, Bach1 represses the expression of heme oxygenase-1 (HO-1), which is one of the major oxidative stress-inducible enzymes protecting cells from oxidative stressors, such as cadmium or 4-hydroxy-2-nonenal treatment [2–6]. Bach1 binds to the multiple Maf recognition elements (MAREs) within the enhancer regions of HO-1 gene under normal conditions [7]. Under oxidative stress, Bach1 protein undergoes nuclear export, allowing the rapid induction of the HO-1 expression by transcriptional activators such as Nrf2 [4,8]. The HO-1 expression is similarly induced by its own substrate, heme, in a Bach1-dependent fashion. Heme binds to Bach1 through its multiple cysteine–proline (CP) motifs, resulting in inhibition of DNA binding, induction of nuclear export, and polyubiquitination and subsequent degradation [9–12].

However, the source of heme that regulates Bach1 has been unclear. In the experiments examining the effect of heme upon Bach1, free heme was added to the culture media [7,9–11]. Such an experimental condition may mimic only a pathological state of heme which is normally present as protein-bound forms. Heme functions as a prosthetic group of hemeproteins (e.g., hemoglobin, myoglobin, catalase and peroxidase) in the aerobic activities of various cells and tissues. In various pathological states, such as vascular hemolysis, rhabdomyolysis, or necrosis, free heme is liberated from hemeproteins into the blood. Extracellular heme may penetrate cell membrane due to its hydrophobicity [13] to affect Bach1. However, blood contains abundant levels of heme-scavenging proteins such as the serum glycoprotein hemopexin (Hx). Hx binds to heme with extremely high affinity ($Ka = 10^{14}\ M^{-1}$ [14]). Heme-bound Hx is subsequently taken up by low-density lipoprotein receptor-related protein 1 (LRP1)-mediated endocytosis [15–17]. Therefore, the majority of heme released into the blood may be chaperoned by Hx to be taken up by cells for not only iron reutilization but also the regulation of Bach1. Thus far, there has been no report on the effect of heme-bound Hx upon Bach1.

Abbreviations: Hx, hemopexin; rHx, recombinant hemopexin; HO-1, heme oxygenase-1; Igκ, immunoglobulin κ; LRP1, low-density lipoprotein receptor-related protein 1; PTM, posttranslational modification; ER, endoplasmic reticulum; CBB, Coomassie Brilliant Blue; CPZ, chlorpromazine; Chx, cycloheximide; PMA, phorbol 12-myristate 13-acetate; 2-ME, 2-mercaptoethanol; LPS, lipopolysaccharide; ROS, reactive oxygen species; Hb, hemoglobin; Hp, haptoglobin

* Corresponding author at: Department of Biochemistry, Tohoku University Graduate School of Medicine, Sendai 980-8575, Japan. Tel.: +81 22 717 7596; fax: +81 22 717 7598.
E-mail address: igarashi@med.tohoku.ac.jp (K. Igarashi).

http://dx.doi.org/10.1016/j.bbagen.2014.02.029
0304-4165/

In most biochemical studies on Hx, native Hx proteins are prepared from the blood of rabbits using multiple steps to remove the many contaminants of major serum proteins, including albumin, transferrin, macroglobulin and immunoglobulin (reviewed in [18]). Recombinant Hx (rHx) proteins have been expressed in *Escherichia coli*, insect cells and *Pichia pastoris* [19–21]. However, it is unclear whether these rHx proteins exhibit properties and functions similar to those of native Hx due to possible differences in glycosylation and the disulfide bond status. Therefore, more suitable systems for obtaining the rHx protein are required to understand the role of Hx in extracellular heme-dependent stress responses.

In this study, we investigated the cellular uptake pathway of extracellular heme and the effects of internalized heme on Bach1 using purified rHx protein expressed in the human cell line, HEK293. To simplify the purification procedures for excluding contaminants, we improved the expression vector and purification methods. First, the signal sequence of Hx was replaced with that of immunoglobulin κ (Igκ) in order to increase the efficiency of secretion [22]. Second, we introduced His-tag into the carboxy-terminus of Hx for affinity purification and immunodetection. Lastly, we generated the HEK293 cell line stably expressing rHx using the FLP–FRT site-specific recombination system [23] in order to constantly obtain the recombinant protein. Using the rHx protein, we compared the effects of rHx-bound heme with those of free heme in terms of the Bach1-regulated HO-1 mRNA expression and found that the former was dependent on endocytosis, while the latter was not. Therefore, our results show that extracellular Hx-bound heme enters cells via Hx-mediated endocytosis, leading to the inactivation of the Bach1 repressor activity and hence the activation of HO-1 gene transcription and oxidative stress defense.

2. Materials and methods

2.1. Plasmids

The Igκ secretion signal sequence was amplified from pDisplay (Invitrogen) using PCR with the following synthetic primers: 5′-AAGCTTACCATGGAGACAGACACACTCCTG-3′ (underline; HindIII site), and 5′-GGATCC CTCGAG GTCACCAGTGGAACCTGG-3′ (underline; BamHI site, double underline; XhoI site). The amplified fragment was subcloned into a pCR-Blunt II-TOPO vector (Invitrogen) and sequenced. The fragment digested with HindIII and BamHI was further ligated into a pcDNA5–FRT vector to generate pcDNA5-Igκ. The 5xHis-tag sequence was introduced into the BamHI site to generate pcDNA5-Igκ-5xHis. Human hemopexin cDNA lacking its signal sequence was amplified using PCR of the cDNA clone of the human hemopexin gene (Open Biosystems). The primer set was 5′-CTCGAGACCCCTCTTCCTCCGACTAG TGCC-3′ (underline; XhoI site) and 5′-GGATCCGTGAGTGCAGCCCAGG AGACTGGT-3′ (underline; BamHI site). The amplified fragment was digested with XhoI and BamHI and subcloned into a XhoI/BamHI-digested pcDNA5-Igκ-5xHis vector to obtain pcDNA5-Igκ-Hx-5xHis. A plasmid expressing a FLAG-tagged mouse Bach1 used in this study has been described previously [24].

2.2. Cell culture

Flp-In 293 cells (Invitrogen) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 10 U/ml of streptomycin and 100 μg/ml of penicillin. THP-1 cells [25] were maintained in RPMI1640 supplemented with 10% FBS, 10 U/ml of streptomycin and 100 μg/ml of penicillin. The THP-1 cells were differentiated into macrophages by adding 10 nM phorbol 12-myristate 13-acetate (PMA) for 24 h. Hepa-1c1c7 cells were maintained in DMEM supplemented with 10% FBS, 10 U/ml of streptomycin and 100 μg/ml of penicillin.

2.3. Antibodies

For the Western blotting analysis, we used the following antibodies: anti-HO-1 (1:1000, Enzo Life Sciences), anti-His-tag (1:1000, Marine Biological Laboratory), anti-FLAG-tag (1:500, Sigma-Aldrich) and rabbit anti-actin antibodies (1:1000, Santa Cruz Biotechnology). The anti-Bach1 antibody has been previously described [9]. The secondary antibodies were horseradish peroxidase-linked anti-rabbit IgG antibodies (1:2000, GE healthcare) or anti-goat IgG antibodies (1:2000, Zymax).

2.4. Establishment of a stable cell line expressing rHx

Flp-In 293 cells were co-transfected with the plasmid pcDNA5-Igκ-Hx-5xHis and a FLP-recombinase vector (pOG44, Invitrogen) in order to generate stable integration of the chimeric hemopexin gene at the FRT-site in the genome. The cells were transfected with 3.6 μg of pOG44 and 0.4 μg of pcDNA5-Igκ-Hx-5xHis using the Gene Juice reagent (Novagen) and further cultured with the complete medium containing the antibiotic hygromycin (300 μg/ml) at 37 °C for one week to select stably rHx-expressing cells.

2.5. Western blotting analysis

Whole cell extracts were prepared as previously described [26]. Lysates or rHx proteins were resolved on SDS-PAGE using 5%–20% polyacrylamide gradient gels (ORIENTAL INSTRUMENTS LTD) and transferred to PVDF membranes (Millipore). The membranes were blocked for 1 h in a blocking buffer [5% skimmed milk in T-TBS buffer (0.05% Tween 20 in TBS)] and subsequently incubated with the primary antibodies in the blocking buffer overnight at 4 °C. After washing with the T-TBS buffer, the membranes were subsequently reacted with the secondary antibodies in the T-TBS buffer for 30 min at room temperature. ECL blotting reagents (Amersham) were used to detect immune complexes.

2.6. Purification of rHx

The stable cells expressing rHx were seeded at 2.0×10^6 cells per 10-cm diameter dishes and cultured for seven days in FreeStyle 293 medium (Invitrogen) with penicillin (100 U/ml) and streptomycin (100 μg/ml) and without FBS. The supernatant was collected and centrifuged at 300 ×*g* for 5 min. The supernatant was diluted 2.5-fold with 20 mM Tris buffer, pH 8.0. Chromatographic purification of rHx was performed using a column with anion exchangers (1 ml HiTrap Q HP, GE Healthcare) and Ni-NTA (1 ml HisTrap, GE Healthcare) on an AKTA purifier system at 4 °C. The anion exchange column absorbed proteins were eluted with a gradually increasing gradient of 20–500 mM NaCl (20 min; flow rate, 1 ml/min) or slowly increasing gradient of 20–300 mM NaCl (25 min; flow rate, 1 ml/min) in 20 mM Tris buffer, pH 8.0. The Ni-NTA column absorbed proteins were eluted with a gradient of 20–500 mM imidazole in 20 mM HEPES buffer, pH 8.0. The fractions containing the rHx protein were confirmed using SDS-PAGE and a Western blotting analysis. The rHx-containing fractions were pooled and concentrated using an Amicon Ultra-30 membrane with a molecular weight cutoff of 30,000 (Millipore). The purity of the rHx protein was estimated using SDS-PAGE.

2.7. Deglycosylation of rHx

The glycosylation status of rHx was evaluated using treatment with the deglycosylation enzymes PNGase F, Endo H_f or o-glycosidase (NEB). After the purified rHx protein was denatured in denaturing buffer (5% SDS, 0.4 M dithiothreitol) with a heating reaction at 100 °C for 10 min, the rHx protein was treated with each deglycosylation enzyme at 37 °C for 1 h according to the manufacturer's protocol (NEB). To deglycosylate the rHx protein under a native state, it was treated

with PNGase F at 37 °C for 12 h without the denaturing step. The deglycosylation of the rHx protein was analyzed using SDS-PAGE.

2.8. Gel filtration assay and absorption analysis of rHx with heme

After 300 μg of the purified rHx protein was incubated with 100 μM heme (dissolved in 100 mM sodium hydroxide and diluted with 100 mM phosphate buffer, pH 7.0) at room temperature for 30 min, the mixture was loaded onto a Superdex 200 (16/60) gel filtration column (GE Healthcare). In each fraction of gel filtration, the heme-bound rHx-containing fractions were identified using an absorption analysis with a ND-1000 NanoDrop spectrophotometer (Thermo Scientific).

2.9. Preparation of rHx-bound heme

rHx-bound heme was prepared via the co-incubation of 50 μM rHx protein and 50 μM heme at room temperature for 20 min. After the reaction, a Bio-spin column (GE Healthcare) was used to exclude free heme and change the buffer to PBS. Finally, the rHx protein or rHx bound with heme was sterilized using an Ultrafree-MC sterile 0.22-μm filter unit (Millipore).

2.10. Quantitative PCR (qPCR)

THP-1 cells were treated with 10 nM PMA and incubated for 24 h in RPMI1640 medium. The differentiated macrophage-like cells were incubated in serum-free RPMI1640 medium containing 1 μM of free heme, 1 μM of heme-bound rHx or 1 μM of rHx for 6 h. The total RNA was purified using the RNeasy Plus Mini kit (Qiagen). A total of 0.2 μg of total RNA was reverse transcribed into cDNA using the Omniscript RT kit according to the manufacturer's protocol using random primers (Invitrogen). The qPCR was performed using the LightCycler (Roche), as previously described [27]. The primer set for the human HO-1 gene was 5′-CCAGCAACAAAGTGCAAG-3′ and 5′-CCACCAGAAAGCTGAGTG TAA-3′. The primer set for β-actin was 5′-AGAGATGGCCACGGCTGCTT-3′ and 5′-ATTTGCGGTGGACGATGGAG-3′. Where indicated, the PMA-treated THP-1 cells were incubated with 5 μg/ml of an endocytosis inhibitor, chlorpromazine (CPZ, Sigma-Aldrich) for 30 min before the addition of heme or heme-bound rHx.

2.11. Inhibition of endocytosis by CPZ

DQ-BSA (Molecular Probes) has been used to measure the endocytic functions [28]. The inhibition of endocytic uptake by CPZ was monitored using DQ-BSA. PMA-induced macrophage-like cells were incubated in 10 μg/ml of DQ-BSA containing PBS with or without 5 μg/ml CPZ for 30 min. The fluorescence of degraded DQ-BSA within each cell was observed using the Leica AF6500 (Leica Microsystems).

2.12. Detection of the HO-1 and Bach1 protein levels

PMA-treated THP-1 cells were incubated in serum-free RPMI1640 medium containing 0–4 μM of heme-bound rHx for 6 h. The cells were lysed with buffer C [20 mM HEPES, pH 7.9, 20% glycerol, 400 mM NaCl, 1 mM EDTA, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM PMSF, 0.1% NP-40, and 1× protease inhibitor cocktail (Roche)] as previously described [26]. HO-1 proteins were detected using a Western blot analysis with anti-HO-1 antibodies. The band intensity of the Western blots was quantified using a densitometric analysis with the Image J software program [29].

2.13. Measuring Bach1 degradation by rHx-bound heme

Hepa-1c1c7 cells and the cells transiently transfected with a plasmid expressing a FLAG-tagged mouse Bach1 using the Gene Juice reagent [24] were individually incubated in serum-free DMEM medium containing 10 μg/ml of cycloheximide (Chx, Wako) and 10 μM of free-heme or 5 μM of heme-bound rHx. Bach1 and HO-1 proteins were detected using a Western blotting assay, as described above. The band intensity of the Western blots was quantified using a densitometric analysis with the Image J software program [29].

3. Results

3.1. Expression and secretion of rHx in the HEK293 cells

To investigate the role of the heme carrier protein Hx in the HO-1 gene regulation, we prepared rHx. Because *E. coli* cells failed to produce rHx capable of binding to heme [19], we used a mammalian expression system considering the functional posttranslational modification (PTM) of secreted proteins. The Hx protein requires PTMs, such as the removal of the signal sequence, and formation of disulfide bonds and glycosylation, in order to bind to heme [19,30,31]. To enhance the secretion capacity, we designed a plasmid containing the human Hx gene that possessed the signal sequence of Igκ instead of the native signal sequence of Hx (Fig. 1A) to achieve its effective secretion [22]. The secreted rHx proteins were detected in the conditioned medium of cells transiently transfected with the expression plasmid pcDNA5-Igκ-Hx-5xHis but not in that of the mock-transfected cells (data not shown).

Next, we took advantage of the Flp-In system to establish cells stably expressing rHx because the transient overexpression of secreted proteins possibly induces endoplasmic reticulum (ER) stress against cells [32]. To exclude contamination from fetal bovine serum, the stable cells expressing rHx were cultured in serum-free FreeStyle 293 expression medium. The expression of rHx from the stable cells was examined using a Western blot analysis against His-tag at the carboxy-terminal end. No bands were detectable in the supernatant of the cells that were stably transfected with the control plasmid pcDNA5-Igκ-5xHis (Fig. 1B, rHx−). In contrast, the rHx proteins were seen as three distinct bands around 60 kDa from that with pcDNA5-Igκ-Hx-5xHis (Fig. 1B, rHx+). Human Hx consists of a single polypeptide chain containing 439 amino acid residues. The molecular weight calculated from the

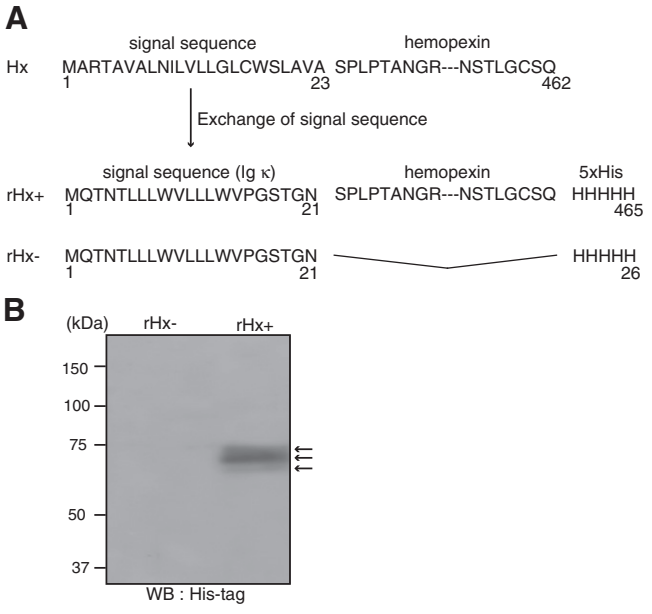

Fig. 1. Expression system of rHx in human cells. (A) The amino acid sequences of human native Hx and rHx used in this study are presented. The secretion signal sequence of native Hx (Hx, 1–23 a.a.) was replaced with that of Igκ (rHx+, 1–21 a.a.). A 5xHis-tag was fused to the carboxy-terminus of Hx. rHx− indicates a construct used as a negative control. (B) The expression of rHx proteins was detected using anti-His-tag antibodies. Multiple bands of rHx proteins were detected around 60 kDa (arrows).

amino acid sequence of the unmodified polypeptide chain is 49 kDa. The difference in the molecular weight of rHx from the theoretical value may be due to PTMs, such as glycosylation. Therefore, the results suggest that the rHx protein secreted from the human cell line was modified by some of the PTMs including glycosylation.

3.2. Purification of rHx

To exclude interfering substances (e.g., Ni chelators) of Ni binding with His-tag, we pretreated the medium of the stable cell line expressing rHx using anion-exchange chromatography. When we eluted the

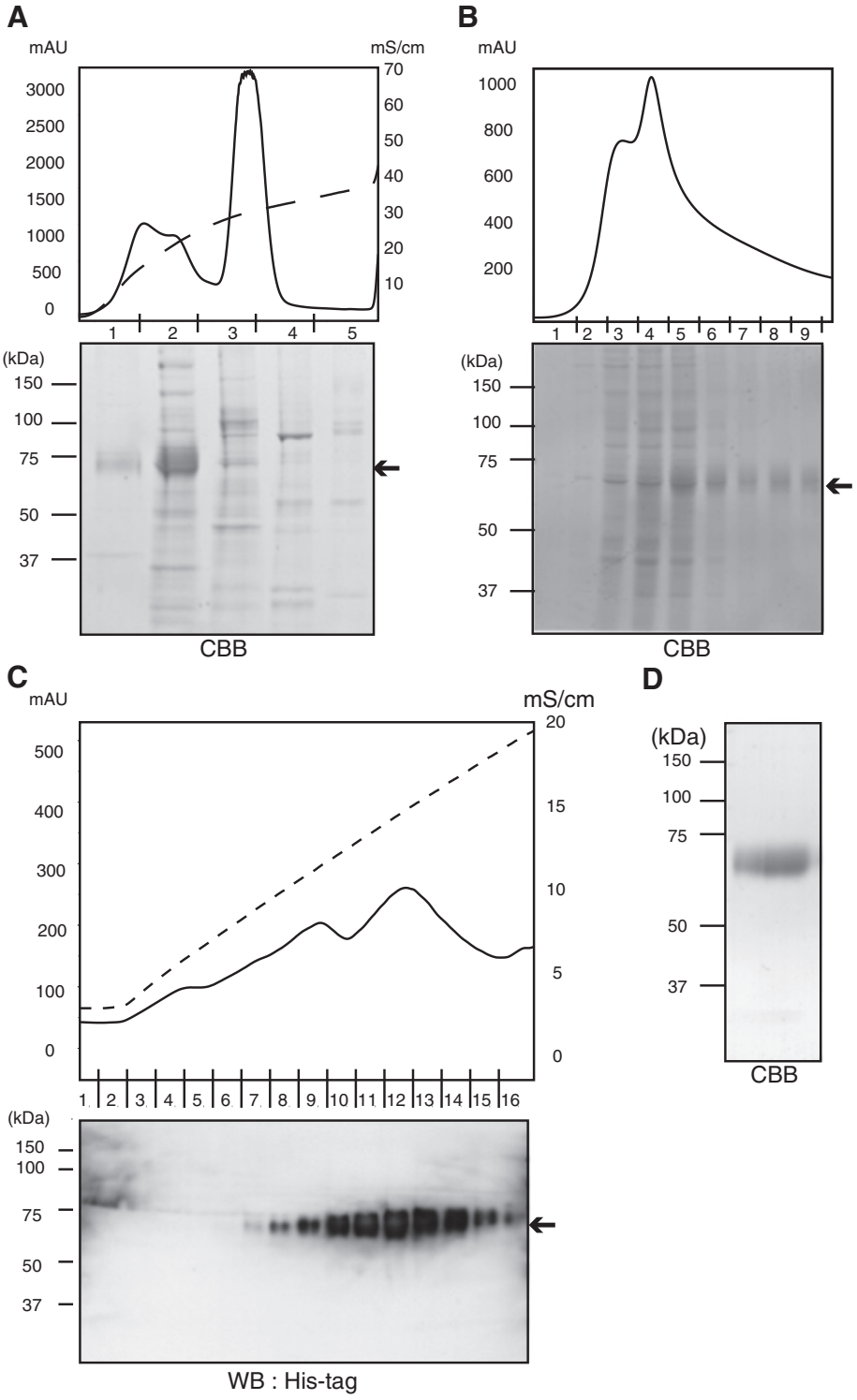

Fig. 2. Purification of rHx proteins from the conditioned medium of the stable cell line. (A) The elution profile of the anion exchange column and the CBB staining of the SDS-PAGE gel (upper and lower panels, respectively) indicate the presence of rHx in fraction 2 (arrow). The solid and dashed lines indicate UV absorption and conductivity, respectively. (B) The elution profile of the Ni column and the CBB staining of the SDS-PAGE gel show the presence but diffuse elution of rHx (arrow). The solid line indicates UV absorption. (C) The elution profile of the anion exchange column and Western blotting using anti-His-Tag antibodies indicate the presence of rHx along two fraction peaks (fractions 9–10 and 11–14, arrow). The solid and dashed lines indicate UV absorption and conductivity, respectively. (D) Purified rHx proteins (15 μg) were separated via SDS-PAGE, and the purity was confirmed using CBB staining.

anion-exchange column-bound proteins with an NaCl concentration gradient (20–500 mM NaCl; 20 min; flow rate, 1 ml/min), the major peak of rHx was observed at fractions 1 and 2 (Fig. 2A). Following anion-exchange chromatography, we further purified the fractions using Ni-affinity chromatography. Nonspecific proteins in the imidazole-gradient fractions were detected on SDS-PAGE, and rHx was broadly eluted along all fractions (Fig. 2B), suggesting that the purification of rHx using Ni-affinity chromatography was inadequate.

Next, we improved the purity of rHx by changing the purification method without using Ni-affinity chromatography. We loaded the medium of the stable cell line onto the anion-exchange column and eluted the column-bound proteins using an NaCl concentration gradient more slowly than before (20–300 mM NaCl; 25 min; flow rate, 1 ml/min). The column-bound rHx protein was eluted from the column at an NaCl concentration between 65 mM and 75 mM (conductivity: 11.5–16.0 mS/cm, Fig. 2C). The fractions 9–14 were identified as rHx-containing fractions using Western blotting with the His-tag antibody (Fig. 2C). The difference in the migration speed of rHx between the two peaks (Fig. 2C, fractions 9–10 and 11–14) may reflect the difference in the glycosylation. The rHx sample was approximately 90% pure, as judged according to Coomassie Brilliant Blue (CBB) staining of SDS-PAGE gel (Fig. 2D), and the yield of purified rHx proteins was approximately 300 μg per 40 ml of the culture medium.

3.3. Characterization of the chemical and biochemical properties of rHx

Previous reports have shown that native human Hx has 12 cysteine residues in six disulfide bridges [30,31,33]. To confirm whether the

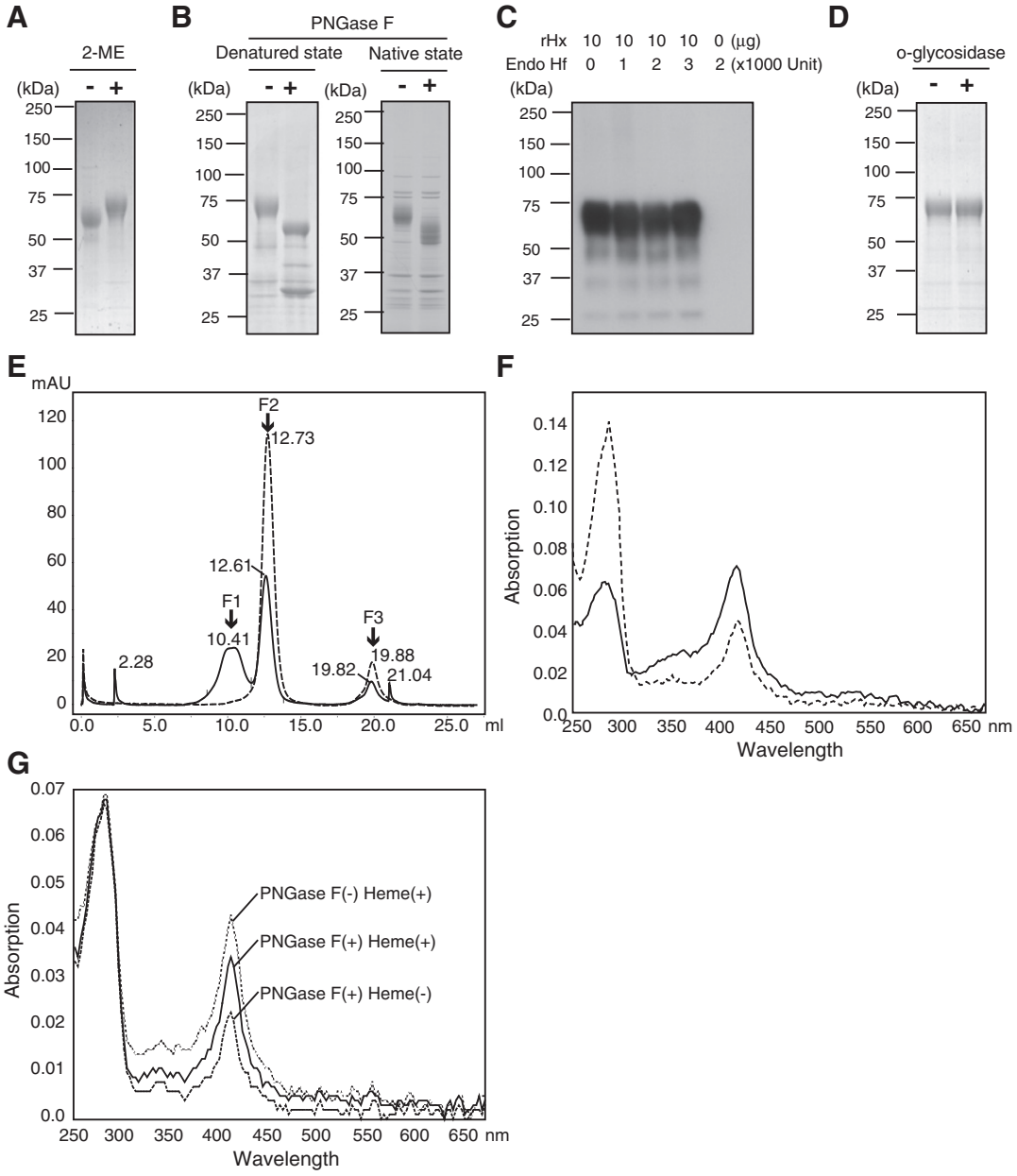

Fig. 3. Posttranslational modification of rHx. (A) The reduction of rHx proteins achieved by adding 2-ME induced slower migration than that observed in the absence of 2-ME. (B) Treatment of rHx proteins with PNGase F under denaturing (left) or native (right) conditions decreased the molecular weight. SDS-PAGE was performed in the presence of 2-ME. (C) Treatment of rHx proteins with Endo H_f did not affect the molecular weight. SDS-PAGE was performed in the presence of 2-ME. rHx was detected using anti-His-tag antibodies. (D) Treatment of rHx proteins with o-glycosidase did not affect the molecular weight. SDS-PAGE was performed in the presence of 2-ME. (E) The gel filtration profiles of rHx and rHx with heme indicate that rHx was separated into peak fractions F2 and F3 (dashed line), while rHx with heme was separated into peak fractions F1, F2 and F3 (solid line). (F) The peak fraction F2 of rHx with heme (solid line) exhibits higher absorbance at 409 nm (Soret peak) than that of rHx without heme (dashed line).

cysteines in purified rHx form disulfide bonds as native Hx, we compared the mobility of the rHx proteins on SDS-PAGE with or without the use of a reducing agent, 2-mercaptethanol (2-ME). The results showed that the reduced rHx proteins migrated more slowly than the non-reduced rHx proteins (Fig. 3A), thus suggesting that rHx was intramolecularly connected by disulfide bonds.

Proper glycosylation is required for the biological activity of Hx, as rHx proteins purified from *E. coli* cannot bind to heme due to a lack of glycosylation [19]. It has been reported that native Hx contains five N-linked and one O-linked oligosaccharides [30,31]. To investigate whether rHx is properly modified by glycosylation, we treated the rHx protein with two types of deglycosylation enzymes. All types of N-linked oligosaccharides are sensitive to PNGase F, whereas only high-mannose oligosaccharides are sensitive to Endo H_f. When the denatured or native rHx protein samples were treated with PNGase F, the molecular weight of rHx decreased from 68 kDa to 55 kDa in both cases (Fig. 3B). The results suggest that 13 kDa of the decrease in molecular weight is due to the removal of N-linked oligosaccharides. To define the pattern of N-linked glycosylation of rHx, we also treated the rHx protein with Endo H_f, which did not affect its mobility on the gels (Fig. 3C). These results show that rHx is glycosylated by complex oligosaccharides. On the other hand, when O-linked oligosaccharides were removed from rHx by o-glycosidase, the mobility of rHx was not significantly affected by the enzyme treatment (Fig. 3D). These results show that rHx is more extensively glycosylated by N-linked oligosaccharides than O-linked oligosaccharides and suggest that the glycosylation and disulfide bond status of rHx is similar to that of native Hx [30,31].

To test the biochemical properties of rHx, we examined the heme-binding activity of rHx according to the absorption spectrum. When native Hx binds to heme, the Soret peak occurs at 409 nm on the absorption spectrum [14,19]. We added heme or buffer to the purified rHx protein and separated the heme-bound rHx from the free rHx using gel filtration. To identify the heme-bound rHx-containing fractions, the absorption spectrum of each fraction was analyzed. Fig. 3E shows the gel filtration chromatogram of rHx and rHx with heme. An F2 peak was observed in two samples, while an F1 peak was observed in only the fraction of rHx with heme (Fig. 3E). When the absorption spectra of the F1 and F2 peak fractions were analyzed, a Soret peak was observed in each F2 fraction (Fig. 3F) but not in the F1 fractions (data not shown). The ratio of the peaks at 409 nm (Soret peak) and 280 nm (protein absorption) of the fractions with heme was higher than that of the fractions without heme (Fig. 3F). These results show that heme-bound rHx exists within the F2 fraction, because a Soret peak of F2 was observed, as in reported data for the native heme–Hx complex [14]. The partial presence of a Soret peak within the fraction without heme addition suggests that a portion of rHx bound heme present in the medium. Therefore, rHx has a similar chemical structure and also similar biochemical properties to those of native Hx. Next, we analyzed whether the glycosylation of rHx was important for heme-binding. Using rHx or rHx de-glycosylated under the native state (see Fig. 3B), we compared their heme-binding activity by measuring the absorption at 409 nm. The results indicated that the heme-binding activity of the de-glycosylated rHx was lower than that of glycosylated rHx (Fig. 3G). Thus, we conclude that the glycosylation of rHx was important for binding to heme.

3.4. Effects of rHx-bound heme on the HO-1 expression

Hx-bound heme is reportedly transported into cells via the endocytic pathway [15], leading to the upregulation of the HO-1 gene expression in hepatocytes [16]. To confirm whether rHx can deliver heme into cells, we measured the HO-1 expression level in a murine hepatoma cell line, Hepa-1c1c7, in the presence or absence of various concentrations of rHx bound with heme. When the Hepa-1c1c7 cells were cultured with heme-bound rHx, the HO-1 protein level increased in a dose- and time-dependent manner (Fig. 4A and B). These results show that rHx-bound heme was incorporated into cells, resulting in the induction of the HO-1 gene expression.

Hx-bound heme is taken up by hepatic cells via LRP1-mediated endocytosis [15,16,34]. LRP1 is expressed not only on hepatocytes but also on macrophages [35]. To investigate whether Hx-bound heme is also taken up by macrophages, we used the human monocytic cell line THP-1. Differentiation of THP-1 cells into macrophage-like cells can be induced by exposing the cells to PMA [25]. When PMA-treated macrophage-like cells were treated with heme-bound rHx, the HO-1 protein level was dose-dependently increased (Fig. 4C). To confirm the dependency upon heme, the PMA-treated cells were treated with rHx alone, heme-bound rHx, or free heme for 0–12 h. Comparing the effect of each component on the HO-1 expression, we observed that free heme and heme-bound rHx, but not rHx alone, induced the HO-1 mRNA expression (Fig. 4D). These results suggest that heme–rHx complex was transferred into the intracellular space and that heme was released from rHx once within the cells, resulting in promotion of the HO-1 expression in hepatocytes and macrophages.

3.5. Investigation of the cellular uptake pathway of heme

A heme-bound Hx scavenging receptor, LRP1, mediates the uptake of various ligands via clathrin-dependent endocytosis [36]. Therefore, in order to investigate whether rHx-bound heme is taken up via endocytosis, we used CPZ, an inhibitor of clathrin-mediated endocytosis [37]. First, to confirm that CPZ inhibits endocytosis-mediated processes, we verified its inhibitory effects on endocytosis using a fluorescent probe, DQ-BSA, that produces fluorescence only when it undergoes lysosomal degradation after endocytosis. We differentiated THP-1 cells into macrophages using PMA and incubated the cells with DQ-BSA in the presence or absence of CPZ. In the absence of CPZ, a weak fluorescence intensity of degraded DQ-BSA was detected within several cells after 10 min. The fluorescence intensity then increased after 20 min (Fig. 5A). In the presence of CPZ, no cells exhibiting fluorescence were detected after 10 min, nor were they significantly detected after 20 min (Fig. 5A). These results confirmed that CPZ inhibited endocytosis.

Next, we examined whether rHx-bound heme is taken up via endocytosis using CPZ. We cultured PMA-treated macrophage-like THP-1 cells with heme-bound rHx or free heme in the presence or absence of CPZ for 6 h. We assessed their uptake by the mRNA levels of HO-1. As expected, the HO-1 mRNA expression was similarly induced by heme-bound rHx and free heme in the absence of CPZ (Fig. 5B, white bars). Importantly, CPZ inhibited the induction of the HO-1 mRNA expression by heme-bound rHx but not by free heme (Fig. 5B, black bars). These results suggest that rHx-bound heme is transferred into cells via LRP1-mediated endocytosis, whereas free heme is taken up by cells via an endocytosis-independent pathway. We could not compare the effect of CPZ at the level of HO-1 protein, because CPZ inhibited protein translation (data not shown). CPZ has been shown to activate unfolded protein response, inhibiting protein translation [38].

3.6. Effects of rHx-bound heme on Bach1 proteins

To investigate the effects of rHx-bound heme on Bach1, we evaluated the Bach1 protein level using the Hepa-1c1c7 murine cell line rather than a human cell line because the anti-Bach1 antibody A1-5 recognizes endogenous murine Bach1, but fails to recognize efficiently human BACH1 [24] (data not shown). When we cultured the Hepa-1c1c7 cells with free heme or heme-bound rHx for 6 h, an increase in the HO-1 protein level was observed in both treatments (Fig. 5C). It is believed that the HO-1 induction was due to Bach1 inactivation. However, we did not find any significant changes in the Bach1 protein level under these conditions (Fig. 5C). One possibility is that the *de novo* synthesis of Bach1 concealed the decrease in Bach1 protein. Therefore, we examined the effects of heme-bound rHx in the presence of Chx. We cultured Hepa-1c1c7 cells with heme-bound rHx along with

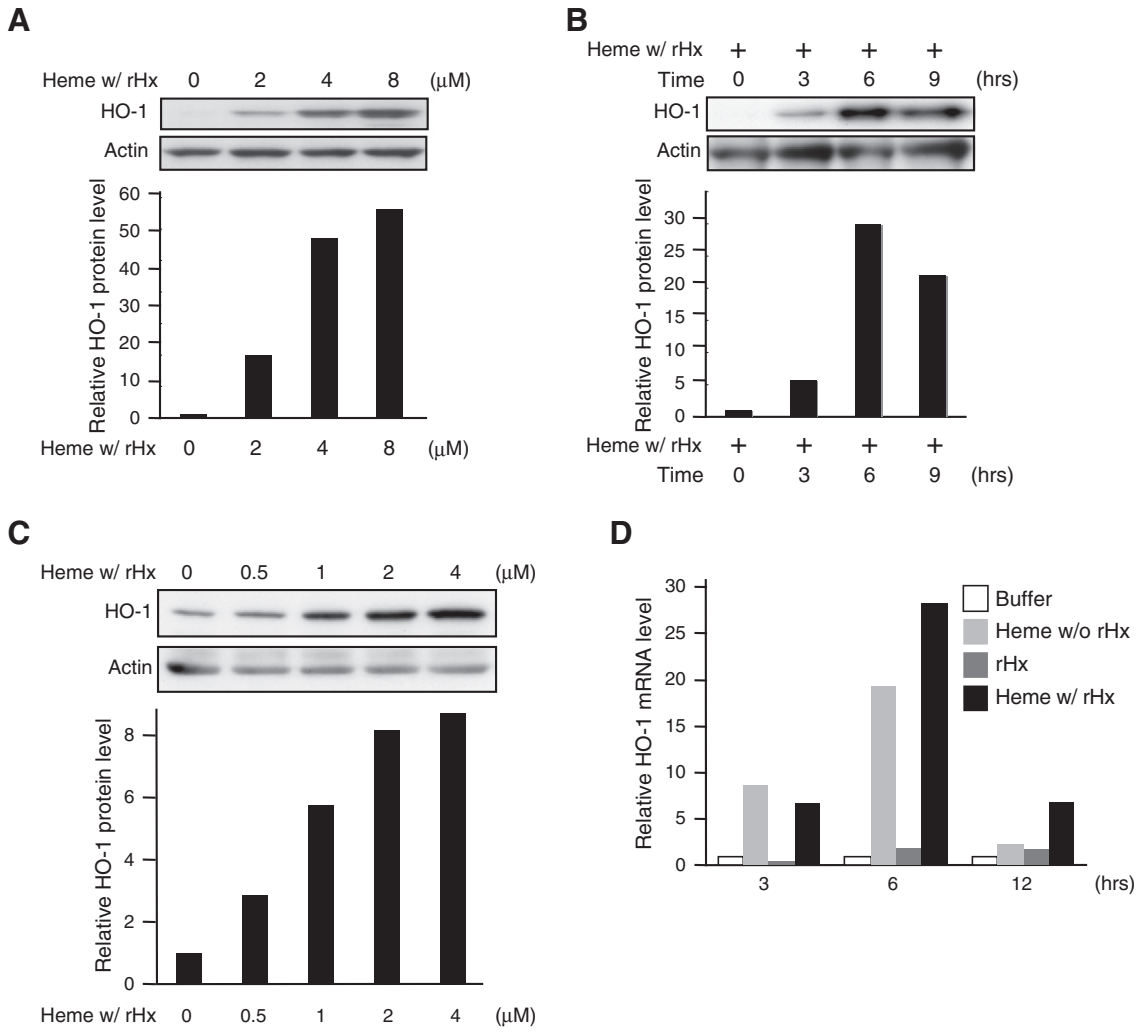

Fig. 4. Effects of rHx-bound heme on the HO-1 expression. (A) The dose-dependent effects of rHx-bound heme on the induction of HO-1 proteins were detected using Western blotting with anti-HO-1 antibodies in Hepa-1c1c7 cells. The cells were cultured with serum-free medium containing 0–8 μM heme-bound rHx for 6 h. The relative HO-1 protein levels were normalized to that of actin and referenced to that of HO-1 without rHx-bound heme. (B) The time-dependent effects of rHx-bound heme on the induction of HO-1 proteins were detected using anti-HO-1 antibodies in Hepa-1c1c7 cells. The cells were cultured with serum-free medium containing 5 μM of rHx-bound heme for 0–9 h. (C) The dose-dependent effects of rHx-bound heme on the induction of HO-1 proteins were detected using anti-HO-1 antibodies in THP-1 cells. The cells were first treated with PMA for 24 h, then cultured with serum-free medium containing 0–4 μM of rHx-bound heme for 6 h. (D) A time-course qPCR analysis of the HO-1 mRNA expression induced by rHx-bound heme and/or its components showed no inducible effects of rHx alone. PMA-treated THP-1 cells were cultured with serum-free medium in which either PBS only (white), 1 μM of heme (gray), 1 μM of rHx (dark gray) or 1 μM of heme-bound rHx (black) was added. The relative HO-1 mRNA levels were normalized to that of actin and referenced to that of HO-1 with buffer at each time point, respectively.

Chx for 0–9 h. Pretreatment with Chx completely abolished the induction of HO-1 proteins (Fig. 5D). This result confirmed that Chx stopped the *de novo* synthesis of proteins. A significant decrease in the Bach1 protein level was induced by heme-bound rHx within 3 h, followed by the disappearance of the Bach1 protein level after 9 h (Fig. 5D, arrowhead). Hence, heme-bound rHx induced a decrease in the level of Bach1 protein. To confirm the effect of heme-bound rHx upon Bach1 protein, we also used Hepa-1c1c7 cells transiently transfected with a plasmid expressing a FLAG-tagged Bach1. After we cultured the cells with heme-bound rHx along with Chx for 0–6 h, we measured the Bach1 protein levels using anti-FLAG-tag antibody. In this situation, heme-bound rHx time-dependently decreased the Bach1 protein levels (Fig. 5E). Taken together, the results suggest that rHx-bound heme upregulates the HO-1 gene expression by inactivating Bach1 as reported for free heme. However, they were different in terms of the dependency upon endocytosis.

4. Discussion

In this study, we developed a method to prepare rHx-bound heme as a major form of extracellular heme in order to investigate the cellular uptake pathway and subsequent effect upon HO-1 gene expression of extracellular heme. We revealed that rHx-bound heme is taken up via endocytosis and that internalized heme acts as a signaling molecule via Bach1 inactivation. Based on our results, the physiological significance of the heme signal will be explored in the future.

Considering the functional importance of PTMs present in Hx, we prepared the rHx protein from mammalian HEK293 cells. Previous reports have shown the importance of the PTM status of Hx for achieving proper biological functions [19,30,31]. When the rHx protein obtained from mammalian cells was treated with PNGase F, the molecular weight of 13 kDa decreased (Fig. 3B), which is equivalent to five N-linked oligosaccharides, as the molecular weight of a typical N-linked oligosaccharide is 2.5 kDa. In contrast, Endo H_f did not affect the molecular weight of rHx (Fig. 3C). These results indicate that rHx is secreted by the addition of mature N-linked oligosaccharides via the ER-Golgi secretory pathway, because Endo H_f acts only on high-mannose oligosaccharides that should be removed in the Golgi apparatus [39]. In addition, when the rHx protein was treated with o-glycosidase, no significant decreases in molecular weight were detected (Fig. 3D). Hx is known to possess an additional O-linked oligosaccharide [30,31], which is equivalent to 0.6 kDa. We

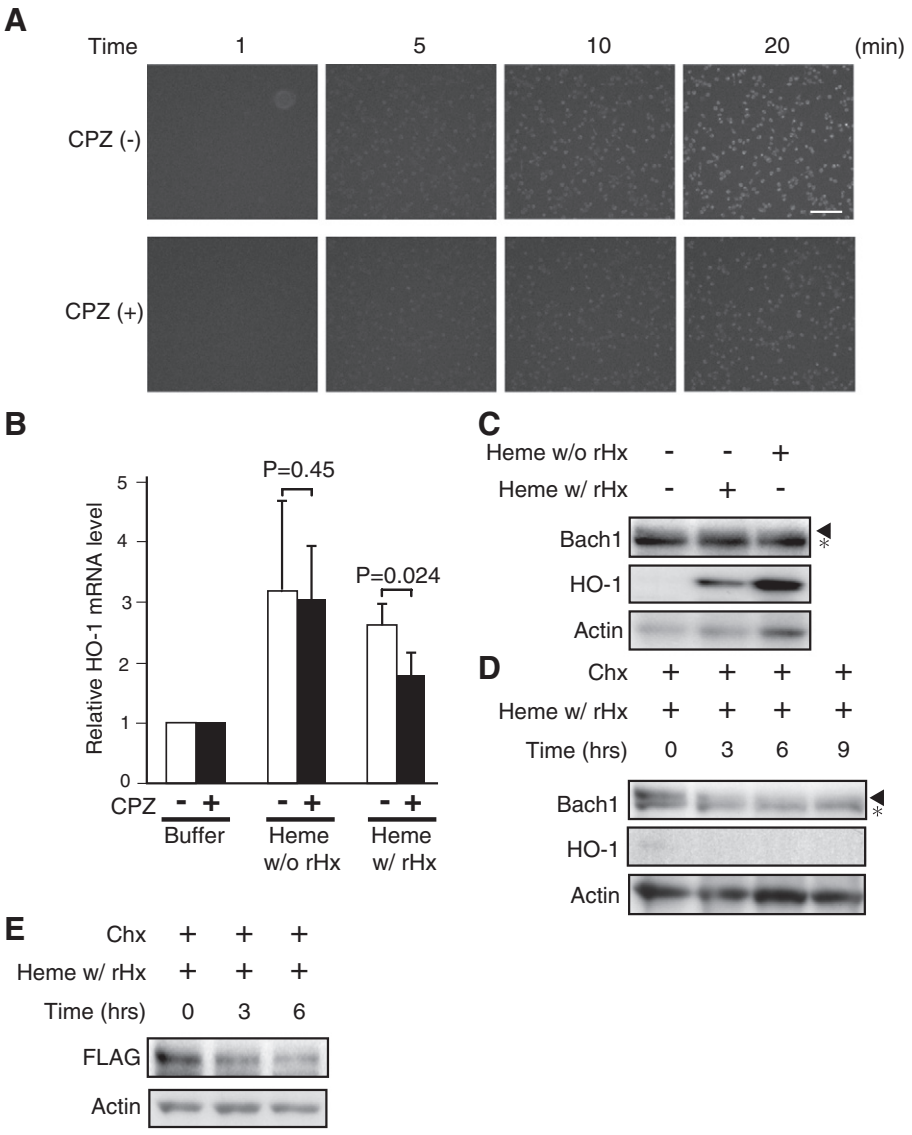

Fig. 5. Investigation of the regulatory pathway of rHx-bound heme with respect to the Bach1 activity. (A) A time-course observation of the fluorescence of degraded DQ-BSA showed the inhibitory effects of CPZ on endocytosis. THP-1 cells were first treated with PMA for 24 h, then cultured with or without CPZ for 30 min. After 10 μg/ml of DQ-BSA was added to the culture medium, the fluorescence within each cell was observed. The scale bar indicates 100 μm. (B) A qPCR analysis of the HO-1 mRNA expression induced by free heme or rHx-bound heme in the presence or absence of CPZ revealed endocytosis-dependency of the HO-1 mRNA expression in THP-1 cells. PMA-treated THP-1 cells were cultured with CPZ for 30 min. After the cells were further treated with 1 μM of heme or 1 μM of rHx-bound heme for 6 h, the HO-1 mRNA was measured using a qPCR analysis. The relative HO-1 mRNA levels were normalized to that of actin and referenced to that of HO-1 with buffer in the presence or absence of CPZ, respectively. *P*-values (Student's *t*-test) for differences between each stimulating agent and the buffer are indicated. (C) The effects of free heme or rHx-bound heme on Bach1 degradation were detected using a Western blot analysis with anti-Bach1 antibodies. After culture medium containing Hepa-1c1c7 cells was added to PBS only, 10 μM of heme or 5 μM of rHx-bound heme, the cells were further cultured for 6 h. (D and E) The time-dependent effects of rHx-bound heme on Bach1 degradation without the *de novo* synthesis of proteins were detected using anti-Bach1 or anti-FLAG-tag antibodies. Hepa-1c1c7 cells (D) or the cells transiently expressing FLAG-tagged Bach1 (E) were cultured with 5 μM of rHx-bound heme in the presence of 10 μg/ml of Chx for the indicated time periods.

cannot exclude the possibility that the difference of 0.6 kDa was undetectable in the SDS-PAGE analysis. Mass spectrometry analyses are needed to draw conclusions on this point. On the other hand, the crystal structure of heme-bound Hx has shown that heme is located near the glycosylation site of Hx [33]. Thus, the possibility that the glycosylation of Hx modulates heme-binding has been proposed but remains unproven. In this study, we revealed that the glycosylation of rHx was important for heme-binding by comparing the heme-binding activity of de-glycosylated rHx with that of glycosylated rHx (Fig. 3G). This result supports the possibility that the structure of Hx is altered by changes in glycosylation status, resulting in modulating the heme-binding.

In this study, we have shown that rHx-bound heme induces the HO-1 mRNA and protein expressions (Fig. 4). As a stress-inducible enzyme, HO-1 plays a critical role in protecting cells from oxidative stress via antioxidant defenses, including the reduction of cellular reactive oxygen species (ROS) generation and the promotion of radical scavenging [13]. While the transcriptional induction of HO-1 mRNA expression involves diverse mechanisms and transcription factors (e.g., Nrf2 and NF-kB) [8,40–42], the inhibition of the repressor activity of Bach1 by heme has been shown to be the critical step for its induction [7,9–12]. Interestingly, tissue damages and cell death lead to release of heme among diverse danger molecules [43]. Therefore, it has implicitly been considered that extracellular heme is delivered into cells to act as a signaling molecule to protect the cells from oxidative stress. Other examples also suggest that extracellular heme functions as a signaling molecule. One of the examples of heme signaling is hemoglobin (Hb). Free Hb is generally bound by haptoglobin (Hp) in the serum. The Hb–Hp complex is sequentially taken up by macrophages via CD163 receptor-mediated endocytosis, leading to the HO-1 expression [44]. Another example is

senescent erythrocytes. The senescent cells are phagocytosed by macrophages in the spleen or bone marrow [45], which induces the expressions of ferroportin1 and HO-1 [46]. This uptake of extracellular heme by diverse pathways may serve to protect cells from oxidative stress via the HO-1 expression.

We showed that rHx-bound heme is taken up by macrophages via endocytosis, while free heme is taken up by an endocytosis-independent pathway (Fig. 5B). The difference in the endocytosis dependency between free heme and rHx-bound heme strongly indicates that these forms of heme induce the HO-1 expression via distinct mechanisms. Taken together with previous data [16], our results (Fig. 5B) show that the endocytosis of rHx-bound heme is mediated by LRP1. It has previously been suggested that the endocytosis of LRP1 attenuates the inflammatory signaling pathways including Toll-like receptor (TLR)-mediated c-Jun NH2-terminal protein kinase (JNK) and nuclear factor-kappa B (NFκB) signaling [47,48]. As these inflammatory signaling pathways are enhanced by free heme [42,49,50], the Hx-mediated endocytosis of LRP1 may suppress the free heme-induced inflammatory signaling. Indeed, Hx significantly downregulates the synergistic induction of proinflammatory cytokines by free heme with LPS [49].

Furthermore, it has been shown that heme without Hx induces both the HO-1 expression and ROS production, while heme bound with Hx induces the HO-1 expression, but not ROS production, in the endothelium [51]. It is believed that Hx protects cells from free heme-mediated ROS production by scavenging extracellular heme and promoting its endocytosis and subsequent degradation. Extracellular free heme promotes the oxidative stress response via the HO-1 gene expression in association with ROS production and NFκB activation. In contrast, Hx transfers extracellular heme into cells via LRP1-mediated endocytosis, leading to the induction of antioxidation programs including the HO-1 expression without the prooxidant and inflammatory effects.

Extracellular materials that are taken up by endocytosis are transferred to lysosomes via endosomes. The interior of the endosome compartment is kept acidic (pH 5–6). Therefore, heme is dissociated from Hx in the endosome [52]. The heme that is liberated from Hx may escape from the endosome or lysosome into the cytoplasm in order to affect the gene expression. The mechanisms by which heme enters into the cytoplasm are largely unknown. Further investigations into the mechanisms by which heme is exported from endosomes or lysosomes into the cytoplasm are required. Recently, heme responsive gene-1 (HRG-1) has been identified to be an endosomal heme transporter [53,54]. We predict that HRG-1 is involved in the exportation of heme from the endosome into the cytoplasm, which will be examined by combining the rHx system and gene knockdown. Taking advantage of the recombinant system, we will be also able to visualize the cellular uptake pathway of extracellular heme using rHx proteins fused with GFP.

We revealed that rHx-bound heme induces the degradation of Bach1 proteins, leading to the expression of HO-1 (Fig. 5C, D, and E). The downregulation of Bach1 plays a role in protection against oxidative stress by derepressing HO-1, which is involved in protecting cells from oxidative stress [6,7,9–12,55,56]. Therefore, Hx-bound heme may promote the antioxidation program by controlling the Bach1 repressor activity without causing free heme-induced prooxidant and inflammatory effects.

It has recently been recommended that Hx be used in the treatment of vasculopathy in patients with hemolytic disorders, stroke and intracerebral hemorrhage [51,57]. The therapeutic activity of Hx may be due to the promotion of the antioxidation program by delivering heme into cells and inactivating Bach1. The heme–Hx–Bach1 axis appears to be clinically important. On one hand, we demonstrated previously that Bach1 ablation reduces the progression of atherosclerosis in cuff injury model mice [58] and the myocardial infarction in myocardial ischemia/reperfusion injury model mice [59]. On the other hand, Hx-deficient mice exhibit recovering defects in acute hemolysis [60]. Based on the fact that the loss of the components of the heme–Hx–Bach1 axis significantly affects the phenotypes under pathological conditions but not normal conditions [1,58–60], the heme–Hx–Bach1 axis is thought to modulate the clinical courses and outcomes of various diseases associated with ROS. It is useful to understand the mechanisms by which extracellular heme is transferred to Bach1 by Hx for the development of preventive treatments for atherosclerosis, acute hemolysis, or other diseases accompanying oxidative and inflammatory effects.

Acknowledgements

We are grateful to Dr. K. Murayama (Tohoku University) for valuable help with the study and for also supplying several reagents. Part of this study was supported by the Biomedical Research Core of the Tohoku University School of Medicine. This work was also supported by Grants-in-aid and the Network Medicine Global-COE Program from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

[1] K. Igarashi, J. Sun, The heme–Bach1 pathway in the regulation of oxidative stress response and erythroid differentiation, Antioxid. Redox Signal. 8 (2006) 107–118.
[2] S. Shibahara, F. Han, B. Li, K. Takeda, Hypoxia and heme oxygenases: oxygen sensing and regulation of expression, Antioxid. Redox Signal. 9 (2007) 2209–2225.
[3] L.E. Otterbein, M.P. Soares, K. Yamashita, F.H. Bach, Heme oxygenase-1: unleashing the protective properties of heme, Trends Immunol. 24 (2003) 449–455.
[4] H. Suzuki, S. Tashiro, J. Sun, H. Doi, S. Satomi, K. Igarashi, Cadmium induces nuclear export of Bach1, a transcriptional repressor of heme oxygenase-1 gene, J. Biol. Chem. 278 (2003) 49246–49253.
[5] M. Ishikawa, S. Numazawa, T. Yoshida, Redox regulation of the transcriptional repressor Bach1, Free Radic. Biol. Med. 38 (2005) 1344–1352.
[6] W. Hou, Q. Tian, N.M. Steuerwald, L.W. Schrum, H.L. Bonkovsky, The let-7 microRNA enhances heme oxygenase-1 by suppressing Bach1 and attenuates oxidant injury in human hepatocytes, Biochim. Biophys. Acta 1819 (2012) 1113–1122.
[7] K. Ogawa, J. Sun, S. Taketani, O. Nakajima, C. Nishitani, S. Sassa, N. Hayashi, M. Yamamoto, S. Shibahara, H. Fujita, K. Igarashi, Heme mediates derepression of Maf recognition element through direct binding to transcription repressor Bach1, EMBO J. 20 (2001) 2835–2843.
[8] K. Itoh, N. Wakabayashi, Y. Katoh, T. Ishii, K. Igarashi, J.D. Engel, M. Yamamoto, Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain, Genes Dev. 13 (1999) 76–86.
[9] Y. Zenke-Kawasaki, Y. Dohi, Y. Katoh, T. Ikura, M. Ikura, T. Asahara, F. Tokunaga, K. Iwai, K. Igarashi, Heme induces ubiquitination and degradation of the transcription factor Bach1, Mol. Cell. Biol. 27 (2007) 6962–6971.
[10] H. Suzuki, S. Tashiro, S. Hira, J. Sun, C. Yamazaki, Y. Zenke, M. Ikeda-Saito, M. Yoshida, K. Igarashi, Heme regulates gene expression by triggering Crm1-dependent nuclear export of Bach1, EMBO J. 23 (2004) 2544–2553.
[11] J. Sun, H. Hoshino, K. Takaku, O. Nakajima, A. Muto, H. Suzuki, S. Tashiro, S. Takahashi, S. Shibahara, J. Alam, M.M. Taketo, M. Yamamoto, K. Igarashi, Hemoprotein Bach1 regulates enhancer availability of heme oxygenase-1 gene, EMBO J. 21 (2002) 5216–5224.
[12] S. Hira, T. Tomita, T. Matsui, K. Igarashi, M. Ikeda-Saito, Bach1, a heme-dependent transcription factor, reveals presence of multiple heme binding sites with distinct coordination structure, IUBMB Life 59 (2007) 542–551.
[13] S. Kumar, U. Bandyopadhyay, Free heme toxicity and its detoxification systems in human, Toxicol. Lett. 157 (2005) 175–188.
[14] Z. Hrkal, Z. Vodrazka, I. Kalousek, Transfer of heme from ferrihemoglobin and ferrihemoglobin isolated chains to hemopexin, Eur. J. Biochem. 43 (1974) 73–78.
[15] A. Smith, W.T. Morgan, Hemopexin-mediated heme uptake by liver. Characterization of the interaction of heme–hemopexin with isolated rabbit liver plasma membranes, J. Biol. Chem. 259 (1984) 12049–12053.
[16] V. Hvidberg, M.B. Maniecki, C. Jacobsen, P. Hojrup, H.J. Moller, S.K. Moestrup, Identification of the receptor scavenging hemopexin–heme complexes, Blood 106 (2005) 2572–2579.
[17] E. Tolosano, S. Fagoonee, N. Morello, F. Vinchi, V. Fiorito, Heme scavenging and the other facets of hemopexin, Antioxid. Redox Signal. 12 (2010) 305–320.
[18] M.R. Mauk, A. Smith, A.G. Mauk, An alternative view of the proposed alternative activities of hemopexin, Protein Sci. 20 (2011) 791–805.
[19] T. Satoh, H. Satoh, S. Iwahara, Z. Hrkal, D.H. Peyton, U. Muller-Eberhard, Roles of heme iron-coordinating histidine residues of human hemopexin expressed in baculovirus-infected insect cells, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 8423–8427.
[20] W.W. Bakker, T. Borghuis, M.C. Harmsen, A. van den Berg, I.P. Kema, K.E. Niezen, J.J. Kapojos, Protease activity of plasma hemopexin, Kidney Int. 68 (2005) 603–610.
[21] R. Lennon, A. Singh, G.I. Welsh, R.J. Coward, S. Satchell, L. Ni, P.W. Mathieson, W.W. Bakker, M.A. Saleem, Hemopexin induces nephrin-dependent reorganization of the actin cytoskeleton in podocytes, J. Am. Soc. Nephrol. 19 (2008) 2140–2149.
[22] M. Horynova, K. Takahashi, S. Hall, M.B. Renfrow, J. Novak, M. Raska, Production of N-acetylgalactosaminyl-transferase 2 (GalNAc-T2) fused with secretory signal Igkappa in insect cells, Protein Expr. Purif. 81 (2012) 175–180.
[23] S. O'Gorman, D.T. Fox, G.M. Wahl, Recombinase-mediated gene activation and site-specific integration in mammalian cells, Science 251 (1991) 1351–1355.

[24] J. Li, T. Shiraki, K. Igarashi, Transcription-independent role of Bach1 in mitosis through a nuclear exporter Crm1-dependent mechanism, FEBS Lett. 586 (2012) 448–454.
[25] S. Tsuchiya, Y. Kobayashi, Y. Goto, H. Okumura, S. Nakae, T. Konno, K. Tada, Induction of maturation in cultured human monocytic leukemia cells by a phorbol diester, Cancer Res. 42 (1982) 1530–1536.
[26] C. Yamasaki, S. Tashiro, Y. Nishito, T. Sueda, K. Igarashi, Dynamic cytoplasmic anchoring of the transcription factor Bach1 by intracellular hyaluronic acid binding protein IHABP, J. Biochem. 137 (2005) 287–296.
[27] A. Muto, K. Ochiai, Y. Kimura, A. Itoh-Nakadai, K.L. Calame, D. Ikebe, S. Tashiro, K. Igarashi, Bach2 represses plasma cell gene regulatory network in B cells to promote antibody class switch, EMBO J. 29 (2010) 4048–4061.
[28] E.S. Mainali, J.G. Tew, Dexamethasone selectively inhibits differentiation of cord blood stem cell derived-dendritic cell (DC) precursors into immature DCs, Cell. Immunol. 232 (2004) 127–136.
[29] C.A. Schneider, W.S. Rasband, K.W. Eliceiri, NIH Image to ImageJ: 25 years of image analysis, Nat. Methods 9 (2012) 671–675.
[30] N. Takahashi, Y. Takahashi, F.W. Putnam, Complete amino acid sequence of human hemopexin, the heme-binding protein of serum, Proc. Natl. Acad. Sci. U. S. A. 82 (1985) 73–77.
[31] N. Takahashi, Y. Takahashi, F.W. Putnam, Structure of human hemopexin: O-glycosyl and N-glycosyl sites and unusual clustering of tryptophan residues, Proc. Natl. Acad. Sci. U. S. A. 81 (1984) 2021–2025.
[32] A.J. Dorner, L.C. Wasley, R.J. Kaufman, Increased synthesis of secreted proteins induces expression of glucose-regulated proteins in butyrate-treated Chinese hamster ovary cells, J. Biol. Chem. 264 (1989) 20602–20607.
[33] M. Paoli, B.F. Anderson, H.M. Baker, W.T. Morgan, A. Smith, E.N. Baker, Crystal structure of hemopexin reveals a novel high-affinity heme site formed between two beta-propeller domains, Nat. Struct. Biol. 6 (1999) 926–931.
[34] E.A. Zemskov, I. Mikhailenko, D.K. Strickland, A.M. Belkin, Cell-surface transglutaminase undergoes internalization and lysosomal degradation: an essential role for LRP1, J. Cell Sci. 120 (2007) 3188–3199.
[35] S.K. Moestrup, J. Gliemann, G. Pallesen, Distribution of the alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein in human tissues, Cell Tissue Res. 269 (1992) 375–382.
[36] R.A. Fuentealba, Q. Liu, J. Zhang, T. Kanekiyo, X. Hu, J.M. Lee, M.J. LaDu, G. Bu, Low-density lipoprotein receptor-related protein 1 (LRP1) mediates neuronal Abeta42 uptake and lysosomal trafficking, PLoS One 5 (2010) e11884.
[37] L.H. Wang, K.G. Rothberg, R.G. Anderson, Mis-assembly of clathrin lattices on endosomes reveals a regulatory switch for coated pit formation, J. Cell Biol. 123 (1993) 1107–1117.
[38] L. De Filippi, M. Fournier, E. Cameroni, P. Linder, C. De Virgilio, M. Foti, O. Deloche, Membrane stress is coupled to a rapid translational control of gene expression in chlorpromazine-treated cells, Curr. Genet. 52 (2007) 171–185.
[39] Y. Ito, Y. Takeda, Analysis of glycoprotein processing in the endoplasmic reticulum using synthetic oligosaccharides, Proc. Jpn. Acad. Ser. B. Phys. Biol. Sci 88 (2012) 31–40.
[40] N. Wijayanti, S. Huber, A. Samoylenko, T. Kietzmann, S. Immenschuh, Role of NF-kappaB and p38 MAP kinase signaling pathways in the lipopolysaccharide-dependent activation of heme oxygenase-1 gene expression, Antioxid. Redox Signal. 6 (2004) 802–810.
[41] T. Ishii, K. Itoh, E. Ruiz, D.S. Leake, H. Unoki, M. Yamamoto, G.E. Mann, Role of Nrf2 in the regulation of CD36 and stress protein expression in murine macrophages: activation by oxidatively modified LDL and 4-hydroxynonenal, Circ. Res. 94 (2004) 609–616.
[42] Y. Lavrovsky, M.L. Schwartzman, R.D. Levere, A. Kappas, N.G. Abraham, Identification of binding sites for transcription factors NF-kappa B and AP-2 in the promoter region of the human heme oxygenase 1 gene, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 5987–5991.
[43] G.B. Fortes, L.S. Alves, R. de Oliveira, F.F. Dutra, D. Rodrigues, P.L. Fernandez, T. Souto-Padron, M.J. De Rosa, M. Kelliher, D. Golenbock, F.K. Chan, M.T. Bozza, Heme induces programmed necrosis on macrophages through autocrine TNF and ROS production, Blood 119 (2012) 2368–2375.
[44] C.A. Schaer, G. Schoedon, A. Imhof, M.O. Kurrer, D.J. Schaer, Constitutive endocytosis of CD163 mediates hemoglobin-heme uptake and determines the noninflammatory and protective transcriptional response of macrophages to hemoglobin, Circ. Res. 99 (2006) 943–950.
[45] J. Vaysse, L. Gattegno, D. Bladier, D. Aminoff, Adhesion and erythrophagocytosis of human senescent erythrocytes by autologous monocytes and their inhibition by beta-galactosyl derivatives, Proc. Natl. Acad. Sci. U. S. A. 83 (1986) 1339–1343.
[46] M.D. Knutson, M.R. Vafa, D.J. Haile, M. Wessling-Resnick, Iron loading and erythrophagocytosis increase ferroportin 1 (FPN1) expression in J774 macrophages, Blood 102 (2003) 4191–4197.
[47] A. Pocivavsek, I. Mikhailenko, D.K. Strickland, G.W. Rebeck, Microglial low-density lipoprotein receptor-related protein 1 modulates c-Jun N-terminal kinase activation, J. Neuroimmunol. 214 (2009) 25–32.
[48] T. Inubushi, A. Kawazoe, M. Miyauchi, Y. Kudo, M. Ao, A. Ishikado, T. Makino, T. Takata, Molecular mechanisms of the inhibitory effects of bovine lactoferrin on lipopolysaccharide-mediated osteoclastogenesis, J. Biol. Chem. 287 (2012) 23527–23536.
[49] T. Lin, Y.H. Kwak, F. Sammy, P. He, S. Thundivalappil, G. Sun, W. Chao, H.S. Warren, Synergistic inflammation is induced by blood degradation products with microbial Toll-like receptor agonists and is blocked by hemopexin, J. Infect. Dis. 202 (2010) 624–632.
[50] T. Shono, M. Ono, H. Izumi, S.I. Jimi, K. Matsushima, T. Okamoto, K. Kohno, M. Kuwano, Involvement of the transcription factor NF-kappaB in tubular morphogenesis of human microvascular endothelial cells by oxidative stress, Mol. Cell. Biol. 16 (1996) 4231–4239.
[51] F. Vinchi, L. De Franceschi, A. Ghigo, T. Townes, J. Cimino, L. Silengo, E. Hirsch, F. Altruda, E. Tolosano, Hemopexin therapy improves cardiovascular function by preventing heme-induced endothelial toxicity in mouse models of hemolytic diseases, Circulation 127 (2013) 1317–1329.
[52] F.I. Rosell, M.R. Mauk, A.G. Mauk, pH- and metal ion-linked stability of the hemopexin–heme complex, Biochemistry 44 (2005) 1872–1879.
[53] A. Rajagopal, A.U. Rao, J. Amigo, M. Tian, S.K. Upadhyay, C. Hall, S. Uhm, M.K. Mathew, M.D. Fleming, B.H. Paw, M. Krause, I. Hamza, Haem homeostasis is regulated by the conserved and concerted functions of HRG-1 proteins, Nature 453 (2008) 1127–1131.
[54] X. Yuan, O. Protchenko, C.C. Philpott, I. Hamza, Topologically conserved residues direct heme transport in HRG-1-related proteins, J. Biol. Chem. 287 (2012) 4914–4924.
[55] H.J. Warnatz, D. Schmidt, T. Manke, I. Piccini, M. Sultan, T. Borodina, D. Balzereit, W. Wruck, A. Soldatov, M. Vingron, H. Lehrach, M.L. Yaspo, The BTB and CNC homology 1 (BACH1) target genes are involved in the oxidative stress response and in control of the cell cycle, J. Biol. Chem. 286 (2011) 23521–23532.
[56] A.K. MacLeod, M. McMahon, S.M. Plummer, L.G. Higgins, T.M. Penning, K. Igarashi, J.D. Hayes, Characterization of the cancer chemopreventive NRF2-dependent gene battery in human keratinocytes: demonstration that the KEAP1-NRF2 pathway, and not the BACH1–NRF2 pathway, controls cytoprotection against electrophiles as well as redox-cycling compounds, Carcinogenesis 30 (2009) 1571–1580.
[57] P. Hahl, T. Davis, C. Washburn, J.T. Rogers, A. Smith, Mechanisms of neuroprotection by hemopexin: modeling the control of heme and iron homeostasis in brain neurons in inflammatory states, J. Neurochem. 125 (2013) 89–101.
[58] S. Omura, H. Suzuki, M. Toyofuku, R. Ozono, N. Kohno, K. Igarashi, Effects of genetic ablation of bach1 upon smooth muscle cell proliferation and atherosclerosis after cuff injury, Genes Cells 10 (2005) 277–285.
[59] Y. Yano, R. Ozono, Y. Oishi, M. Kambe, M. Yoshizumi, T. Ishida, S. Omura, T. Oshima, K. Igarashi, Genetic ablation of the transcription repressor Bach1 leads to myocardial protection against ischemia/reperfusion in mice, Genes Cells 11 (2006) 791–803.
[60] E. Tolosano, S. Fagoonee, E. Hirsch, F.G. Berger, H. Baumann, L. Silengo, F. Altruda, Enhanced splenomegaly and severe liver inflammation in haptoglobin/hemopexin double-null mice after acute hemolysis, Blood 100 (2002) 4201–4208.