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Invited Review

The Heme Oxygenase Dilemma in Cellular Homeostasis: New Insights for the Feedback Regulation of Heme Catabolism

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SHIBAHARA, S. *The Heme Oxygenase Dilemma in Cellular Homeostasis: New Insights for the Feedback Regulation of Heme Catabolism.* Tohoku J. Exp. Med., 2003, **200** (4), 167–186 —— Heme must be synthesized and degraded within an individual nucleated cell. Heme degradation is catalyzed by the two isozymes of heme oxygenase, heme oxygenase-1 (HO-1) and HO-2, eventually yielding biliverdin/bilirubin, CO, and iron. These products possess important physiological roles but are potentially toxic to cells. Characteristically, human HO-1 contains no Cys residues, whereas HO-2 contains the potential heme-binding motifs of the Cys-Pro dipeptide. Expression of HO-1 is inducible or repressible, depending on cell types or cellular microenvironments, but expression levels of HO-2 are fairly constant. Thus, the main regulation of heme catabolism is a problem of the balance between induction and repression of HO-1. Notably, HO-1 expression is induced by heme in all mammalian cells examined, but is repressed by hypoxia in certain types of cultured human cells. The recent discovery of Bach1 as a heme-regulated and hypoxia-inducible repressor for transcription of the HO-I gene has provided a missing link in the feedback control of heme catabolism. On the other hand, the human HO-1 gene promoter contains the (GT)n repeat polymorphism and a single nucleotide polymorphism (−413A ⟶ T), both of which may contribute to fine-tuning of the transcription. Importantly, long (GT)n alleles are associated with susceptibility to smoking-induced emphysema or coronary artery disease, but may provide with resistance to cerebral malaria. The latter finding suggests a novel therapeutic strategy with inhibitors of HO-1 for the treatment of cerebral malaria. We discuss the potential regulatory role of Bach1 and HO-2 in heme catabolism and update the understanding of the regulation of HO-1 expression. ——
Bach1; bilirubin; hypoxia; iron; malaria.

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All nucleated cells depend on heme, an iron-protoporphyrin, for their survival, as heme senses or uses oxygen (Fig. 1). Heme functions as a prosthetic moiety of various hemoproteins such as hemoglobin, myoglobin, and cytochromes. Thus, heme must be synthesized and degraded within an individual cell, because heme cannot be recycled among different cells (Fig. 2). A well-known exception to this rule is seen in senescent erythrocytes that are phagocytosed by macrophages in the reticuloendothelial system, in which hemoglobin is separated to the heme and globin moieties. Human erythrocytes have an average life-span of about 120 days in the circulation, and thus a large number of erythrocytes must be renewed every day. In macrophages, heme derived from hemoglobin is broken down by heme oxygenase, and the globin moiety is hydrolyzed to amino acids.

Many investigators are interested in the induction of heme oxygenase and its implications, and many excellent reviews have been published (Agarwal and Nick 2000; Immenschuh and Ramadori 2000; Soares et al. 2001; Durante 2003; Otterbein et al. 2003). In this article, however, we rather focus on the repression of heme oxygenase expression, as this subject has been largely ignored. In addition, we present an overview of heme catabolism by focusing on its feedback regulation and interindividual variations. These topics are also discussed in a comprehensive review (Sassa and Shibahara 2003 and refer-

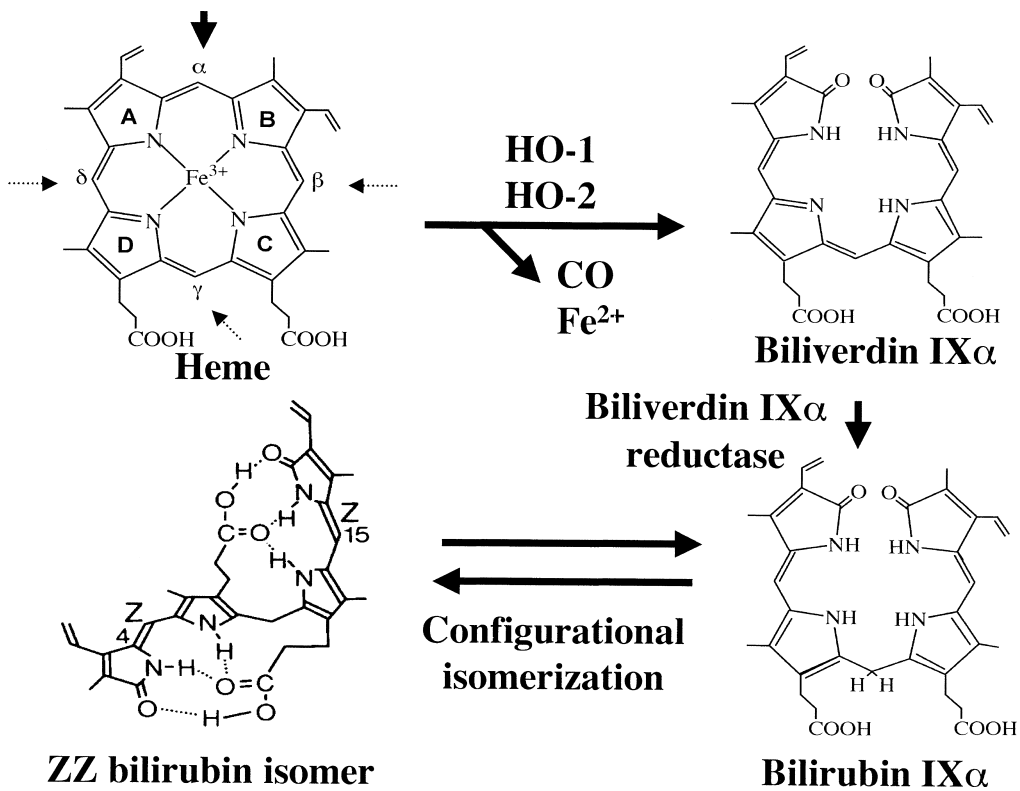

Fig. 1. Structures of heme and bile pigments. Note that only the α-methene bridge of heme is cleaved by HO-1 or HO-2, indicated with a bold arrow. Other biliverdin isomers (β, γ, and δ isomers) could be produced by an unknown mechanism (small arrows). Biliverdin IXα is reduced to bilirubin IXα by biliverdin IXα reductase. Both biliverdin IXα and bilirubin IXα are linear tetrapyrrole compounds. Note the unique conformation of bilirubin IXα (ZZ isomer), in which rings A and B lie in one plane, and rings C and D lie in another.

ences therein) and in a minireview (Sibahara et al. 2003).

A short history of the heme oxygenase

Heme oxygenase was discovered in the late 1960s by Rudi Schmid and colleagues (Tenhunen et al. 1968, 1969). They detected the enzyme activity in the microsomal fraction of rat spleen, liver and kidney that catalyzes the oxidative degradation of heme to biliverdin IXα, carbon monoxide (CO) and iron (Fig. 1). Biliverdin IXα is a straight-chain tetrapyrrole and is rapidly reduced to bilirubin IXα by biliverdin IXα reductase (Tenhunen et al. 1970b). Under physiologic conditions, heme oxygenase activity is higher in the tissues such as the spleen, liver and bone marrow, where senescent erythrocytes are sequestered and degraded. In humans, the daily production of bilirubin IXα is between 250 and 400 mg, and about 80% of bilirubin IXα is accounted for by the degradation of hemoglobin heme. Bilirubin IXα exhibits a hydrophobic property due to the formation of intramolecular hydrogen bonds involving the two propionic acid side chains (Fig. 1). Consequently, intrauterine fetuses may easily excrete bilirubin IXα than biliverdin IXα through placenta into maternal circulation (McDonagh et al. 1981).

Notably, heme oxygenase activity is inducible in cultured cells or in experimental animals by treatment with hemin (Tenhunen et al. 1970a; Pimstone et al. 1971) or with a number of nonheme substances (Gemsa et al. 1974; Maines and Kappas 1974). Thus, heme oxygenase has attracted particular attention of many researchers. Yoshida and Kikuchi (1978) purified the heme oxygenase from pig spleen and characterized its catalytic features. The availability of the purified enzyme allowed us to prepare specific antibody and to show that the heme-mediated induction is associated with the increased heme oxygenase protein and its translatable mRNA in primary culture of pig alveolar macrophages (Shibahara et al. 1978, 1979).

Heme oxygenase isozymes: Two enzymes are better than one

In 1985, heme oxygenase cDNA was cloned from the rat spleen using a specific antibody as a probe (Shibahara et al. 1985). Subsequently, a second form of heme oxygenase was discovered in the rat testis (Maines et al. 1986; Trakshel

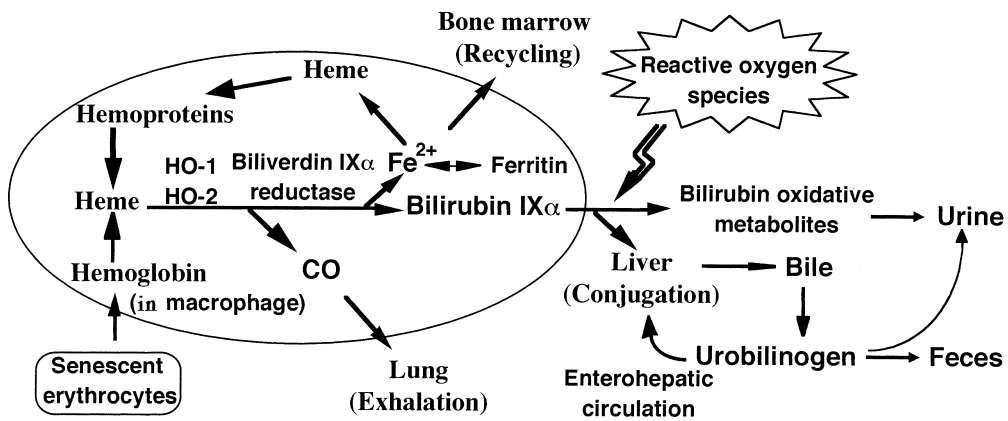

Fig. 2. Heme catabolism in mammals. In a typical nucleated cell, heme is derived from turnover of hemoproteins and is cleaved by heme oxygenase (either HO-1 or HO-2) to generate CO, biliverdin IXα and ferrous iron. Reduction of biliverdin IXα to bilirubin IXα represents the final step of the heme breakdown reaction. Iron is used for heme synthesis in the cell or transported via transferrin to other tissues, mainly the bone marrow. Bilirubin IXα is transported to the liver, where bilirubin IXα is conjugated and excreted into bile. CO is transported to the lung and exhaled. Note that hemoglobin derived from senescent erythrocytes is a major source of heme in macrophages.

et al. 1986). The newly discovered enzyme was termed heme oxygenase-2 (HO-2), and the classic enzyme is now referred to as heme oxygenase-1 (HO-1). The putative isozyme, HO-3, was reported in the rat brain by cDNA cloning, but its enzyme activity was not detected, despite that it shares 90% identity with HO-2 (McCoubrey et al. 1997b). Further studies are required to establish the identity of putative HO-3.

Human HO-1 and HO-2 share 43% amino acid sequence identity (Yoshida et al. 1988; McCoubrey et al. 1992; Ishikawa et al. 1995) (Fig. 3). The human HO-1 and HO-2 genes are localized to chromosome 22q12 and to 16p13.3, respectively (Kutty et al. 1994; Kuwano et al. 1994). Human HO-1 is composed of 288 amino acids with a molecular mass of 33 kDa and shares 80% amino acid sequence identity with rat HO-1 (Yoshida et al. 1988). HO-1 lacks a signal peptide, but contains a hydrophobic segment of 22 amino acid residues at the carboxyl terminus (Fig. 3), which may be important for the insertion of HO-1 into the endoplasmic reticulum. It is noteworthy that either human or rat HO-1 contains no cysteine residues, whereas human HO-2 consists of 316 amino acids with a molecular mass of 36 kDa, and contains three cysteine residues (for review Shibahara 1994). HO-2 contains the extended amino-terminus and a hydrophobic domain at its carboxyl terminus. Notably, unlike HO-1, HO-2 contains two copies of the heme-binding site, a dipeptide of cysteine and proline (CP motif), which are not involved in heme breakdown reaction (McCoubrey et al. 1997a). These CP motifs are conserved at equivalent positions of human (Ishikawa et al. 1995), mouse (accession # AF029874, reported by Mount D.B.), and rat HO-2 (McCoubrey et al. 1992). It is therefore conceivable that HO-2 may serve to sequester heme to maintain the intracellular heme level or reduce heme-mediated oxidative stress (Fig. 4).

A novel role of HO-2 in heme homeostasis

Like HO-2, erythroid type δ-amino levulinic acid synthase contains the CP motifs (Lathrop and Timko 1993), suggesting that heme itself may regulate not only its degradation but also its synthesis (for review Sassa and Nagai 1996). In addition, expression of HO-2 mRNA was not noticeably changed in human cells under various conditions, in which HO-1 expression was remarkably induced (Shibahara et al. 1993; Takeda et al. 1994; Takahashi et al. 1996, 1997). Relatively constant expression levels of HO-2 may be suitable for a regulatory role of HO-2 in heme homeostasis.

HO-2 deficient mice are fertile and survive

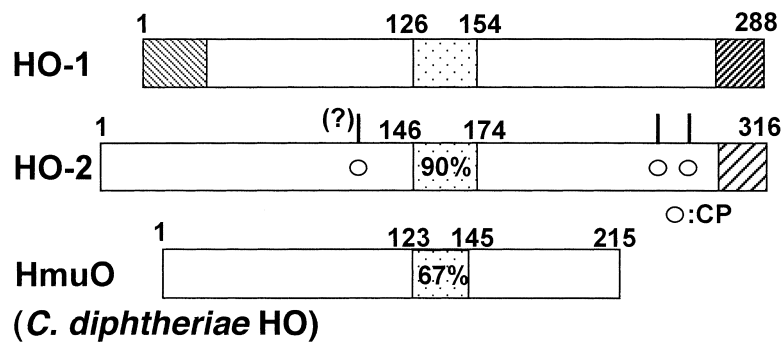

Fig. 3. Structures of heme oxygenase proteins. The conserved catalytic domain is shown as stippled, and the number indicates the amino acid sequence identity to human HO-1. HmuO represents heme oxygenase of *Corynebacterium (C.) diphtheriae*. Human HO-2 contains three copies of CP motif, and one of them, marked with (?), is not conserved in mouse and rat HO-2. In contrast, human HO-1 and HmuO contain no Cys residues.

normally for at least one year (Poss et al. 1995), and thus show milder phenotypes than those of HO-1 deficient mice, such as infertility and prenatal lethality (Poss and Tonegawa 1997). There are no noticeable morphological alterations in HO-2 deficient (-/-) mice, except that cerebral HO activity was markedly reduced. Subsequent studies revealed that ejaculatory abnormalities and reduced mating behavior in male HO-2 (-/-) mice (Burnett et al. 1998) and increased susceptibility to hyperoxic lung damage (Dennery et al. 1998). Thus, the function of HO-2 is not necessarily compensated by HO-1. Interestingly, expression of HO-1 was increased by two fold in the HO-2 (-/-) lungs (Dennery et al. 1998) but not changed in the HO-2 (-/-) brains (Poss et al. 1995). Moreover, the cellular heme levels did not change in the brain and other tissues (Zakhary et al. 1997) and the lung of HO-2 (-/-) mice (Dennery et al. 1998). These results suggest the presence of a backup system that maintains the intracellular heme level.

Features of heme degradation by heme oxygenase

The heme molecule contains four potential cleavage sites, but only α-methene bridge is cleaved by HO-1 and HO-2 (Fig. 1). The HO-1 and HO-2 proteins share the properties in the substrate specificity and cofactor requirements (Maines 1997). Each heme oxygenase protein requires reduced form of nicotinamide-adenine dinucleotide phosphate (NADPH), molecular oxygen and NADPH-cytochrome P450 reductase to cleave heme. Heme oxygenase binds heme at an equimolar ratio and exhibits regiospecific catalytic activities by expending three molecules of oxygen and at least seven electrons provided by NADPH-cytochrome P450 reductase (Yoshida and Kikuchi 1978; for review Yoshida and Migita 2000). The crystal structure of the heme-human HO-1 complex provides evidence that steric regulation is responsible for regiospecific cleavage of α-methene bridge of heme (Schuller et al. 1999).

The heme degradation products, bilirubin IXα, CO, and ferrous iron, are important bioactive molecules. CO represents a direct marker for heme catabolism, while CO gas in the environment is mainly produced during the incomplete combustion of carbon-based fuels. CO is bound to hemoglobin to form carboxyhemoglobin, which is transported to the lungs and is excreted in exhaled air (Fig. 2). Iron is transported to the entire tissues, especially bone marrow, and is reutilized for erythropoiesis and heme biosynthesis. CO has received much attention because of its physiological functions similar to those of NO. The biologic significance of CO, such as anti-inflammatory, anti-apoptotic and anti-proliferative roles, has been summarized in recent review articles (Kajimura et al. 2002; Otterbein et al. 2003).

Bilirubin IXα as a chain-breaking antioxidant and a photoreactive molecule

Bilirubin IXα, which is produced in all tissues, is transported as a non-covalent complex with albumin to the liver, where bilirubin IXα is conjugated with a glucuronide moiety and excreted into bile (Fig. 2). In healthy subjects, serum bilirubin concenrations are determined by its production and hepatic metabolism of bilirubin IXα (uptake, conjugation, or biliary excretion). Bilirubin IXα has been considered as a toxic waste product, because neonatal hyperbilirubinemia could cause bilirubin encephalopathy, also known as kernicterus. The primary choice of treatments for severe neonatal hyperbilirubinemia is phototherapy that depends on the chemical property of bilirubin IXα and effectively prevents neurological complications (for review Sassa and Shibahara 2003). On the other hand, the protective role of bilirubin IXα has been established as a radical scavenger (Stocker et al. 1987) and a chain-breaking antioxidant, as judged by the production of bilirubin oxidative metabolites (Yamaguchi et al. 1994b). Thus, the induction of HO-1 reflects the defense against oxidative stress. In the subsequent sections of this article, bilirubin

represents bilirubin IXα unless otherwise specified.

It is noteworthy that human fetal bile at 20 weeks of gestation contains four possible bilirubin isomers, comprising 6% bilirubin IXα, 87% bilirubin IXβ, 6% bilirubin IXδ and 0.5% bilirubin IXγ (Blumenthal et al. 1980; Yamaguchi et al. 1994a; Yamaguchi and Nakajima 1995). Moreover, adult human bile contains bilirubin IXα (more than 95%) and a small amount of bilirubin IXβ (3–5%) (Yamaguchi and Nakajima 1995). Bilirubin IXβ can be excreted directly into the bile without conjugation, because unlike bilirubin IXα, bilirubin IXβ does not form internal hydrogen bonding. It is unknown how biliverdin IXβ and other isomers are produced in fetus. In this context, there is an enzyme, biliverdin IXβ reductase, which is able to reduce three biliverdin isomers (β, γ, and δ isomers) to respective bilirubin isomers (see Fig. 1), but not biliverdin IXα (Yamaguchi et al. 1994a). Interestingly, biliverdin IXβ is identical to NAD(P)H-linked flavin reductase, which is predominantly expressed in erythrocytes and may function as a methemogblobin reducing enzyme (Komuro et al. 1996; Shalloe et al. 1996). Thus, variety exists in the heme breakdown reaction and remains to be investigated.

Iron is sweet for some bacterial pathogens

The content of body iron is strictly regulated, and iron released from heme is effectively reused for heme biosynthesis. However, in the presence of iron, superoxide anion radical and hydrogen peroxide can be involved in the formation of hydroxyl radical. The physiological importance of HO-1 was confirmed by the severe phenotypic consequences of the HO-1 deficient mice (Poss and Tonegawa 1997) and a patient with HO-1 deficiency (Yachie et al. 1999). The first case of HO-1 deficiency was a compound heterozygote, with a complete deletion of exon 2 of the maternal allele and a two-base deletion within exon 3 of the paternal allele (Fig. 4). These findings have established that HO-1 is required for iron reutilization and that the induction of HO-1 represents a defense mechanism to protect cells from oxidative damage. The implications of the HO-1 deficient patient were extensively discussed in a recent review (Shibahara et al. 2002).

Iron is an essential requirement for most bacteria. Some bacterial pathogens are able to uptake iron by oxidative cleavage of the host heme. *Corynebacterium diphtheriae*, a gram-positive aerobic bacterium, possesses HmuO protein, which shares 33% identity with human HO-1 (Schmitt 1997). This bacterium is the causative agent of diphtheria and produces a virulent diphtheria toxin under the regulation of iron. Unlike mammalian HO-1, HmuO protein lacks the hydrophobic C-terminus and functions as a soluble heme degradation enzyme. HmuO has the active site structure similar to HO-1 and HO-2 (Fig. 3) and shows a similar mechanism of heme degradation (Chu et al. 1999). Subsequently, the heme oxygenase proteins were identified in a gram-negative pathogen *Neisseria meningitides* (Zhu et al. 2000) and in *Pseudomonas aeruginosa*, a common causative agent of opportunistic infections (Ratliff et al. 2001). The HemO protein of *Neisseria meningitides* shows 21% amino acid identity to human HO-1. The pigA gene of *Pseudomonas aeruginosa* encodes a heme oxygenase protein that shares 37% identity with HemO protein. Notably, the PigA protein produces biliverdin IXβ, indicating that this protein shows the regiospecificity of heme cleavage that is different from HO-1 and HO-2.

Bach1 as a missing link in heme homeostasis

Members of the small Maf family (MafK, MafF, and MafG) are basic region leucine zipper (bZip) proteins that can function as transcriptional activators or repressors (Igarashi et al. 1995; Motohashi et al. 2000). In 1996, Igarashi and colleagues have identified two novel transcription factors, Bach1 and Bach2, as heterodimerization partners of MafK (Oyake et

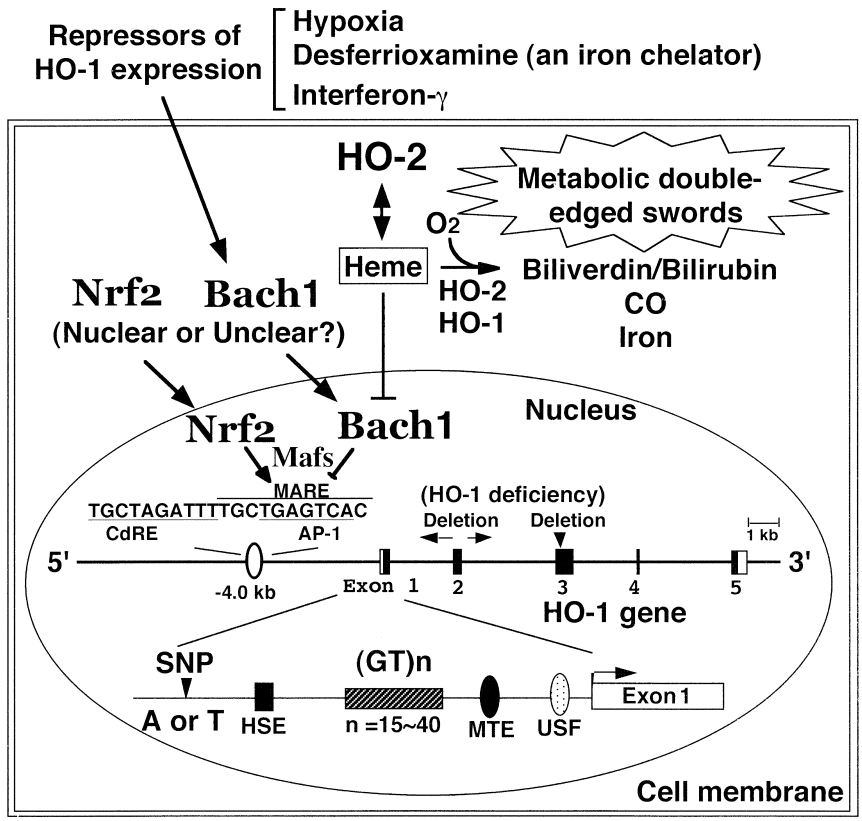

Fig. 4. A dilemma of the human HO-1 gene in its transcriptional regulation. Note a feedback regulation involving HO-1, Bach1, and heme in a nucleated cell (shown as a double-lined rectangle). HO-2 may sequester heme to maintain the intracellular level of free heme. The composite enhancer of the human HO-1 gene, containing cadmium-responsive element (CdRE) and a putative Maf recognition element (MARE), are schematically shown. The putative MARE may be bound by heterodimers, consisting of Nrf2 or Bach1 and one of small Maf proteins. Also shown are potential heat shock element (HSE), the polymorphic (GT)n repeat, and two E box motifs in the proximal promoter region (Sato et al. 1990; Muraosa and Shibahara 1993). The two mutant HO-1 alleles are indicated (Yachie et al. 1999).

al. 1996). These Bach proteins possess a bZip domain and a BTB/POZ domain that has been shown to be involved in the regulation of chromatin structure (Fig. 5). Bach1 and Bach2 show significant similarity to each other in these regions but are otherwise divergent (overall 38% identity).

The Bach1-small Maf heterodimer could repress transcription of target genes by binding to the Maf recognition element (MARE) (Oyake et al. 1996; Igarashi et al. 1998). Importantly, the DNA binding activity of Bach1 is negatively regulated by direct Bach1-heme interaction, suggesting Bach1 as a heme-regulated transcriptional repressor (Ogawa et al. 2001). In fact, Bach1 of 739 amino acid residues has six copies of the CP motif, some of which were bound by heme in vitro. Furthermore, Bach1 is widely expressed in mouse and human tissues that have been analyzed (Oyake et al. 1996; Kanezaki et al. 2001), whereas expression of Bach2 is restricted to monocytes and neuronal cells. Bach1 is induced in cultured human cells by hypoxia, interferon-γ or desferrioxamine, each of which represses HO-1 expression (Kitamuro et al. 2003). Bach1 therefore may function as a controller of the feedback regulation by sensing heme and repress or activate transcription depending on the heme availability (Fig. 5).

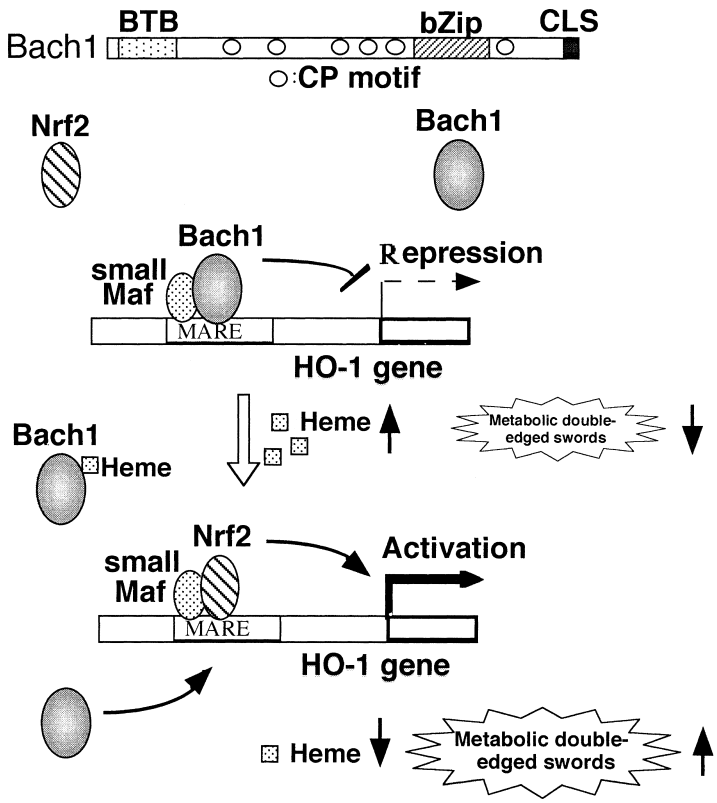

Fig. 5. The Bach1 dilemma in transcriptional regulation of the HO-1 gene. The structure of Bach1 is schematically shown top. Note a functional competition for a small Maf protein between Bach1 and Nrf2 (Sun et al. 2002). CLS stands for cytoplasmic localization signal.

The human HO-1 gene contains at least one copy of functional MARE immediately downstream from the cadmium-responsive element (Kitamuro et al. 2003). Recently, a novel enhancer internal to the human HO-1 gene has been reported (Hill-Kapturczak et al. 2003). The entire 12.5 kb of the human HO-1 gene, including introns and exons, in conjunction with a −4.5-kb promoter region conferred on the reporter gene significant heme- and cadmium-mediated induction. This enhancer function is orientation independent and requires a region between −3.5 and −4.5 kb of the human HO-1 promoter, in which the MARE is located. Thus, the MARE is also important in transcriptional activation of the HO-1 gene; namely, Nrf2 or other related factors could be responsible for the regulation of chromatin structure, thereby allowing the collaboration between the upstream and downstream enhancers.

Bach1-deficient mice are fertile and appear to survive normally (Sun et al. 2002). Surprisingly, HO-1 is constitutively over-expressed in many tissues of the Bach1-deficient mouse (Sun et al. 2002), indicating that Bach1 acts as a negative regulator of transcription of the HO-1 gene in the mouse. Importantly, the inter-tissue difference in the regulation of HO-1 expression has been shown in the Bach1 and Nrf2 compound-deficient mice and may be a consequence of the differential expression levels of relevant transcriptional regulators. In this context, transcription of the mouse HO-1 gene is activated through MAREs by heterodimers, which consist of Nrf2, a factor related to nuclear factor erythroid 2, and one of small Maf proteins (Alam et al. 2000; Ishii et al. 2000). Thus, availability of Nrf2 and Bach1 may determine whether transcription of the HO-1 gene is activated or repressed in various tissues (Itoh et

al. 1997; Sun et al. 2002).

Bach1 is a good candidate that may act on the locus control region (LCR) to repress transcription of the β-globin gene (Igarashi et al. 1998; Yoshida et al. 1999). Bach1 repressed the enhancer activity of the LCR in a BTB/POZ domain-dependent manner. Bach1 expression is induced during megakaryocytic differentiation of $CD34^+$ cells from human cord blood induced by thrombopoietin and during erythroid differentiation induced by erythropoietin (Terui et al. 2000). It would be of interest to analyze the hematopoiesis in Bach1-deficient mice.

Repression of HO-1 expression in cultured human cells

It is not necessarily easy to evaluate whether induction of HO-1 is beneficial or harmful to the host, as the heme degradation products function as metabolic double-edged swords. HO-1 expression is repressed in cultured human cells under thermal stress (Okinaga et al. 1996) or hypoxia (Nakayama et al. 2000; Kitamuro et al. 2003), or by the treatment with interferon-γ (Takahashi et al. 1999a) or an iron chelator, desferrioxamine (Nakayama et al. 2000; Kitamuro et al. 2003). It should be noted, however, that hypoxia induced expression of HO-1 in human dermal fibroblasts (Panchenko et al. 2000) and unaffected its expression in explants of normal human chorionic villi from term placentas (Appleton et al. 2003), suggesting the inter-tissue difference in the regulation of HO-1 expression. Importantly, these repressors for human HO-1 expression could induce HO-1 expression in cultured rodent cells. We therefore hypothesize that the repression of HO-1 expression may represent a defense strategy developed in humans (Shibahara et al. 2002), although experimental findings in cultured human cells do not necessarily reflect the human condition. Accordingly, we have been interested in the significance of the (GT)n repeat polymorphism in the HO-1 gene promoter, as discussed later.

The intracellular level of heme is regulated by the rate of its synthesis and degradation. Heme is synthesized by collaboration of eight enzymes and their regulators (Furuyama and Sassa 2000; for review Fujita 1997; Sassa and Shibahara 2003); namely, heme is an expensive (invaluable) molecule. In fact, the heme catabolism mediated by HO-1 or HO-2 results in the production of bilirubin IXα, CO, and ferrous iron, all of which are important bioactive molecules. Expression levels of HO-1 or HO-2 may also affect the intracellular heme pool, which in turn influences the availability of heme for the synthesis of various hemoproteins. For example, overexpression of HO-1 via retrovirus gene transfer reduced the levels of heme and cGMP in pulmonary microvessel endothelial cells (Abraham et al. 2002), the levels of prostaglandin E2 (Quan et al. 2002), and the cyclooxygenase activity (Haider et al. 2002; Quan et al. 2002). These results suggest that the degree of HO-1 expression modulates the levels of cellular heme, which in turn may influence the activities of soluble guanylate cyclase and cyclooxygenase.

The repression of HO-1 expression is important in the feedback regulation mediated by intracellular heme (Figs. 4 and 5). Namely, the repression may transiently increase the intracellular heme levels, which may facilitate heme to bind to Bach1, thereby derepressing transcription of the target genes of Bach1 (Ogawa et al. 2001). In addition to the secondary effects on hemoproteins, there are two physiological consequences in the repression of HO-1 expression. Its repression reduces energy expenditure consumed for oxidative heme breakdown, and prevents the local accumulation of CO, iron, and bilirubin IXα beyond certain threshold levels in the HO-1-expressing cells and their surroundings (Figs. 4 and 5). On the other hand, the repression could restrict iron supply to cancer cells or certain pathogens, such as bacteria and protozoa, that might be carried by a host, because HO-1 is responsible for the

turnover of iron that is an essential requirement for cell proliferation. In fact, expression levels of HO-1 and HO-2 mRNAs were higher in eight cases of excised primary brain tumors than those in control brain tissue (Hara et al. 1996).

HO-1 is not a heat shock protein in humans: No induction is good news

The proximal promoter region of the rat HO-1 gene contains the functional heat shock element (HSE) (Müller et al. 1987; Sato et al. 1989; Okinaga and Shibahara 1993), and HO-1 mRNA expression and activity are increased in rat cells by heat shock (42°C) (Shibahara et al. 1987). Moreoever, hyperthermia was shown to lead to the remarkable induction of HO-1 mRNA and protein in the rat brain (Ewing and Maines 1991). Thus, rat HO-1 has been established as a heat shock protein (HSP32). Likewise, the proximal promoter region of the human HO-1 gene contains an HSE (Fig. 6), but HO-1 mRNA expression and activity are not inducible by heat shock in many types of human cells (Shibahara et al. 1989; Okinaga et al. 1996). Moreover, heme oxygenase activity is not induced by heat shock in cultured cells derived from human, monkey, pig, and mouse, but is induced by hemin treatment in all the cells examined (Shibahara 1988), indicating the interspecies difference in the regulation of HO-1 expression by heat shock.

It has been established that HO-1 is a stress-response protein in human cells (Yoshida et al. 1988; Keyse and Tyrrell 1989; Taketani et al. 1989). However, we have been interested in the lack of HO-1 induction by heat shock (42°C) in human cells, as fever is an evolutionary conserved response in the host defense and essentially beneficial to the host. We then provided evidence that the sequence flanking the HSE may prevent the heat-mediated activation of the human HO-1 gene (Okinaga et al. 1996). Such a silencing effect is of particular significance in the brain, because heme degradation products possess potential toxic effects. If the human HO-1 gene lacks such an element of silencing activity, HO-1 is easily induced in the human brain under various conditions, as seen in the rat brain (Ewing and Maines 1991). The human HO-1 gene has gained the silencer sequence to protect its harmful induction by heat shock, which may be related to the defense against certain diseases, such as malaria. Fever in malaria (40°C or more) is schizontocidal but contributes to synchronization of parasites' life cycle within erythrocytes, thereby generating the characteristic fever spikes (Kwiatkowski and Nowak 1991).

An SNP in the human HO-1 gene promoter

The two reported genomic clones of the human HO-1 gene were derived from DNA of different individuals (Shibahara et al. 1989; Takeda et al. 1994). Sequence comparison of these HO-1 promoters revealed the two sequence differences in their promoter regions (Fig. 6): polymorphic (GT)n repeats and the A/T polymorphism ($-413A \rightarrow T$) (Takahashi et al. 1999b). The (GT)n repeats of the first and second clones are the $(GT)_{15}AT(GT)_{14}$ and $(GT)_{23}$, respectively. The former repeat of $(GT)_{15}AT(GT)_{14}$ is categorized as a $(GT)_{30}$ allele. The single nucleotide difference (A or T) is not due to sequencing error or cloning artifacts, as either A or T was found in several genomic samples. It is therefore most likely that the difference represents an SNP. This SNP may contribute to fine-tuning of the HO-1 gene transcription. It remains to be investigated whether each SNP generates the binding site for a separate transcription factor (Fig. 6).

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-540 TAAATGTACA TTTAAAGAGG GTGTGAGGAG GCAAGCAGTC AGCAGAGGAT TCCAGCAGGT

-480 GACATTTTAG GGAGCTGGAG ACAGCAGAGC CTGGGGTTGC TAAGTTCCTG ATGTTGCCCA

                *
-420 CCAGGCTATT GCTCTGAGCA GCGCTGCCTC CCAGCTTTCT GGAACCTTCT GGGACGCCTG
         SNP                                   HSE
-360 GGGTGCATCA AGTCCCAAGG GGACAGGGAG CAGAAGGGGG GGCTCTGGAA GGAGCAAAAT

-300 CACACCCAGA GCCTGCAGCT TCTCAGATTT CCTTAAAGGT TTTGTGTGTG TGTGTGTGTG

-240 TGTGTGTGTG TGTATGTGTG TGTGTGTGTG TGTGTGTGTG TGTTTTCTCT AAAAGTCCTA
          (GT)n repeats
-180 TGGCCAGACT TTGTTTCCCA AGGGTCATAT GACTGCTCCT CTCCACCCCA CACTGGCCCG
                                  MTE
-120 GGGCGGGCTG GGCGCGGGCC CCTGCGGGTG TTGCAACGCC CGGCCAGAAA GTGGGCATCA

 -60 GCTGTTCCGC CTGGCCCACG TGACCCGCCG AGCATAAATG TGACCGGCCG CGGCTCCGGC
                     USF          TATA-like
   *
   1 AGTCAACGCC TGCCTCCTCT CGAGCGTCCT CAGCGCAGCC GCCGCCCGCG GAGCCAGCAC
                                  exon 1
  61 GAACGAGCCC AGCACCGGCC GGATGGAGCG TCCGCAACCC GACAGGCAAG CGCGGGGC
                                                    intron 1
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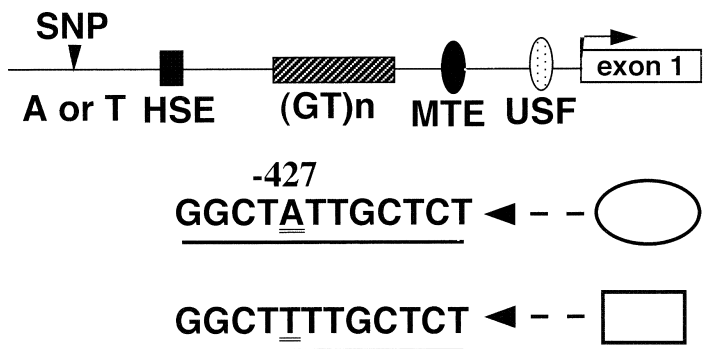

Fig. 6. Updated nucleotide sequence of the human HO-1 gene promoter. Shown is the nucleotide sequence of the HO-1 genomic clone (Shibahara et al. 1989) (accession number: X14782). The second clone contains a T residue at position −413 (based on the numbering of the first clone) and the $(GT)_{23}$ repeats (Takahashi et al. 1999b; accession number: AF145047). Two types of the sequences near the SNP site are highlighted at the bottom and might be bound by separate transcription factors (shown as square and oval).

Microsatellite polymorphism in the HO-1 gene promoter

Transcription of the HO-1 gene is under the regulation of the fine-tuning system that includes the Bach1 system and the (GT)n spacer polymorphism (Fig. 6). Notably, the polymorphic (GT)n repeats are not present at the equivalent positions of the rat HO-1 gene (Müller et al. 1987). Analyses of Japanese people have revealed that the numbers of (GT)n repeats vary from 15 to 40 (Kimpara et al. 1997), and two common repeats are 23 and 30 (Yamada et al. 2000). The long (GT)n repeats are likely to form Z-DNA, left-handed helix, and may reduce transcription from the HO-1 gene promoter. Thus, the (GT)n repeat may influence the basal promoter activity or the induction level of the HO-1 gene expression in response to stress. It is conceivable that a new variant repeat in the HO-1 gene became fixed or stabilized in a population by providing the individual with a selective advantage, depending on environmental conditions.

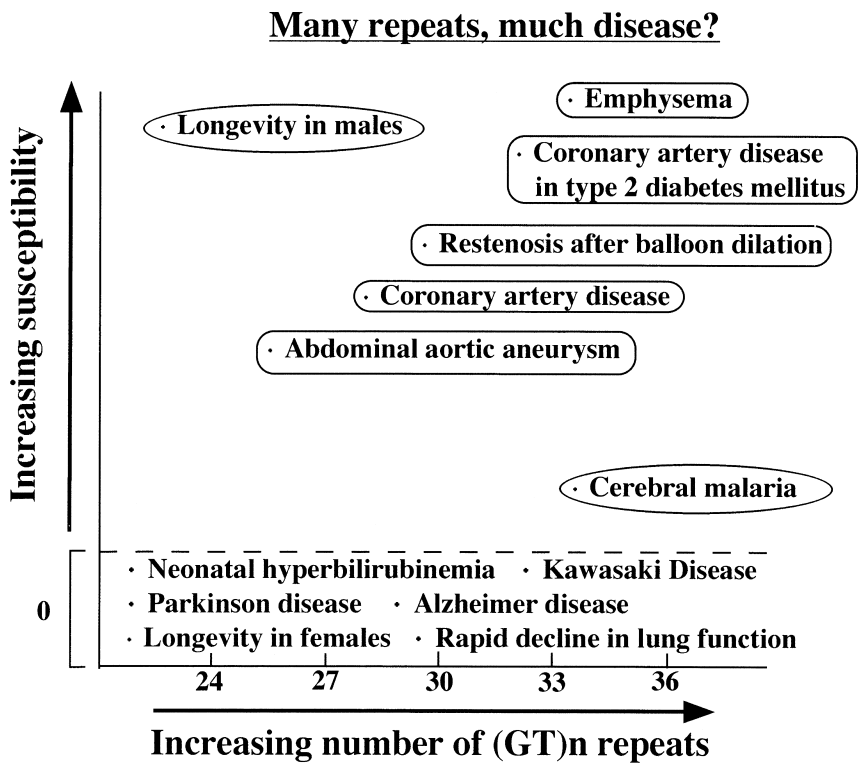

Fig. 7. Many repeats, much disease. Potential roles of (GT)n repeats in the HO-1 gene promoter in susceptibility to some diseases are schematically shown. The indicated diseases are arbitrarily allocated, depending on the numbers of (GT)n repeats, but their positions do not necessarily reflect the degrees of susceptibility. The diseases, shown below the broken line, are not associated with any particular (GT)n repeat alleles (indicated with 0). Note that long (GT)n repeats may provide protection against cerebral malaria.

Long GT alleles (>32) in the HO-1 gene promoter were shown to be associated with susceptibility to pulmonary emphysema (Yamada et al. 2000). Probably, the short (GT)n alleles are associated with higher HO-1 expression levels, and bilirubin or CO may be protective against emphysema caused by cigarette smoking. Accordingly, the functional consequences of the polymorphisms were analyzed in established lymphoblastoid cell lines, which were derived from subjects possessing S/S (<27 GT) or L/L genotypes (>32 GT) (Hirai et al. 2003). HO-1 mRNA expression and HO activities induced by oxidative stress were significantly higher in the lymphoblastoid cell lines with S/S genotypes than those with L/L. Furthermore, the cell lines with S/S genotypes were more resistant to oxidant-induced apoptosis than those with L/L. These findings suggest that the short alleles of the HO-1 gene confer the antiapoptotic effects and more resistant to oxidative stress-mediated diseases.

To date, the associations with GT repeat polymorphism have been investigated in various diseases (Fig. 7). In most cases, long (GT)n alleles are associated with susceptibility to the pathological conditions, including restenosis within the 6 months after transluminal angioplasty in the femoropopliteal segment (Exner et al. 2001), coronary artery disease in diabetic (Chen et al. 2002) or non-diabetic patients (Kaneda et al. 2002), and abdominal aortic aneurysm (Schillinger et al. 2002). In individuals with long (GT)n repeats, the induction of HO-1 is not sufficient to protect the vascular

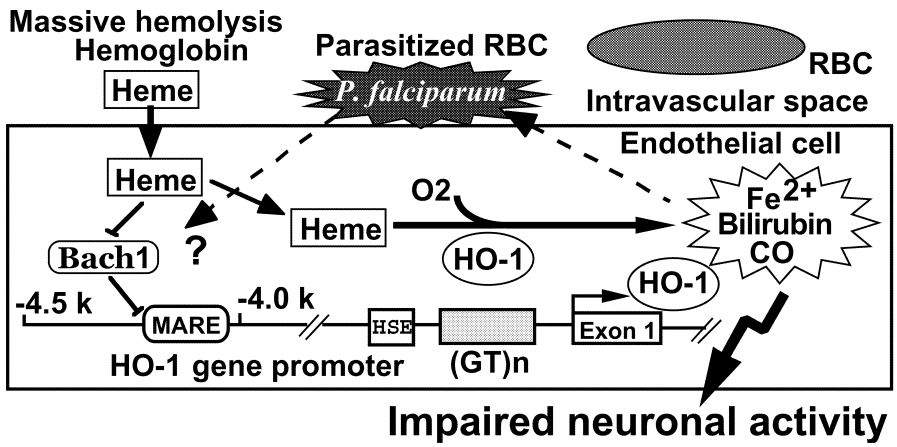

Fig. 8. Hypothetical roles of HO-1 in the pathogenesis of cerebral malaria. Shown are the potential cross-talk between an endothelial cell of the brain microvasculature and a parasitized erythrocyte that is sequestered on the endothelial cell. RBC stands for red blood cells. Note the altered membrane properties of parasitized erythrocytes. Patients with cerebral malaria show massive intravascular hemolysis. Heme, derived from endogenous hemoproteins (not shown) and blood, must be actively degraded in endothelial cells and other cell types. Likewise, hemoglobin derived from hemolysis may induce HO-1 in macrophages. In addition, *P. falciparum* parasites within erythrocytes may activate the transcription of the HO-1 gene through a hitherto unknown mechanism, shown as a broken arrow. The heme degradation products may impair neuronal activity, while they, especially iron, are reused by parasites within erythrocytes.

tissue damage or inhibit vascular smooth muscle cell proliferation (for review Durante 2003). Longevity in males is inversely related to long GT alleles (Yamaya et al. 2003).

In contrast to the above vascular diseases that affect the elderly, no significant association with any GT repeat polymorphism was found in patients with Kawasaki disease (Kanai et al. 2003b), which is an acute systemic vasculitis of unknown etiology and affects infant and children (Kawasaki et al. 1974). Kawasaki disease is frequently complicated with coronary aneurysm. The difference may reflect the pathogenesis of Kawasaki disease and other vascular diseases for the elderly.

No significant associations were found in neonatal unnconjugated hyperbilirubinemia (Kanai et al. 2003a), Alzheimer and Parkinson diseases (Kimpara et al. 1997), longevity in females (Yamaya et al. 2003), and rapid decline in lung function (He et al. 2002). It should be noted that even the negative results do not exclude the involvement of HO-1 in the pathogenesis of these diseases.

Beneficial role of long GT alleles in cerebral malaria

Surprisingly, Hirayama (2003) has reported that long (GT)n repeats ($n>30$) in the HO-1 gene promoter are associated with a lower incidence of cerebral malaria in Karen ethnic groups who live near the border between Myanmar and Thailand (Fig. 7). Apparently, there is a need for larger studies of the relation between cerebral malaria and microsatellite polymorphism in areas of different endemicity. This finding, however, suggests that heme degradation products may be involved in the pathogenesis of cerebral malaria. Malaria (bad air in Italian) is a worldwide protozoan infection, and severe malaria caused by *Plasmodium falciparum* leads to 1–2 million deaths of children (under 5 years of age) each year. Severe malaria manifests as hemolytic anemia, jaundice, and coma. The pathogenesis of coma, known as cerebral malaria, is related to the

adherence of *falciparum*-infected red blood cells through "knobs" to vascular endothelium of the cerebral microvasculature (Fig. 8), thereby leading to local hypoxia (Miller 1994; for review Shibahara et al. 2002 and references therein). In this context, the hypoxic repression of HO-1 may be beneficial to malaria patients, as growth and proliferation of *P. falciparum* within erythrocytes depend on iron supply from microenvironments. An apparent weak point of *falciparum* parasites is that they are unable to cleave heme to release iron. Ironically, the parasites must uptake the iron from blood or vascular wall, despite they reside in hemoglobin-rich erythrocytes.

It is crucial for the host to maintain the iron status at a level that does not facilitate availability of iron to pathogens, as already discussed in some pathogenic bacteria. In fact, treatment with desferrioxamine, a repressor of human HO-1 expression, was reported to be effective for cerebral malaria in Zambian children (Gordeuk et al. 1992, 1995). It is therefore conceivable that iron supply to parasitized erythrocytes, from vascular wall at and near the sequestered site in the brain, is limited in the individuals with longer (GT)n repeats (Fig. 8). In malaria patients who usually exhibit severe anemia and massive hemolysis, iron dynamics Rave been set toward its transportation to the bone marrow for erythropoiesis. Thus, iron is released from endothelial cells and macrophages to blood rather than to be stored in the cells as ferritin. Conversely, the shorter (GT)n repeats may be associated with efficient induction of HO-1, which results in the release of larger amounts of CO, iron and bilirubin from vascular endothelial cells at the sites of sequestration. In fact, the induction of HO-1 mRNA is associated with production of bilirubin IXα in the vascular wall (Nakayama et al. 2001). The heme degradation products may alter the neuronal activity in the brain, while some of the heme degradation products, especially iron, may be supplied to parasites within erythrocytes. Accordingly, long (GT)n repeats may provide resistance to cerebral malaria.

Concluding remarks

Humans had successfully adapted to upright bipedal walking, which must be accompanied by the physical and anatomical changes in the cardiovascular, respiratory, and nervous systems. The current findings suggest the inter-species, inter-individual, and inter-tissue variations in the regulation of the HO-1 gene expression. Such variations may reflect the defense strategy uniquely developed in humans, as evident from the polymorphic (GT)n repeat in the human HO-1 gene promoter.

Future studies will be aimed to explore the possibility whether HO-2 or Bach1 may function as an oxygen sensor. Detailed analyses of HO-2- or Bach1-deficient mice may provide us with invaluable information concerning heme or oxygen homeostasis. But we must be careful to use the rat or mouse models for the study of stress response, including HO-1 expression. For example, most rodents are able to produce vitamin C (ascorbic acid), unlike humans and primates (Challem and Taylor 1998). In addition, there are no rodent models for cerebral malaria, although rodent malaria may be fatal (Shibahara et al. 2002 and references therein).

Induction or downregulation of HO-1 expression in human cells by pharmacological means will be a promising strategy for the treatment of various disorders. Perhaps, induction or over-expression of HO-1 may be helpful for some vascular diseases listed in this article (Fig. 7). On the other hand, the potential protective role of the long (GT)n alleles in cerebral malaria provides exciting therapeutic implications, as the development of vaccine is still underway and drug-resistant *falciparum* strain is common. It is therefore urgent to develop an effective treatment of cerebral malaria. Tin-protoporphyrin and Tin-mesoporphyrin are potent inhibitors for heme oxygenase and have been successfully used for treatment of hyper-

bilirubinemia in newborns without serious complications (Sassa 1997). Thus, it is tempting to speculate the application of metalloporphyrins for treatment or prevention of cerebral malaria.

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