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Review

Lead, Chemical Porphyria, and Heme as a Biological Mediator

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FUJITA, H., NISHITANI, C. and OGAWA, K. *Lead, Chemical Porphyria, and Heme as a Biological Mediator*. Tohoku J. Exp. Med., 2002, **196** (2), 53–64 —— One of the most well-characterized symptoms of lead poisoning is porphyria. The biochemical signs of lead intoxication related to porphyria are δ-aminolevulinic aciduria, coproporphyrinuria, and accumulation of free and zinc protoporphyrin in erythrocytes. From the 1970s to the early 80s, almost all of the enzymes in the heme pathway had been purified and characterized, and it was demonstrated that δ-aminolevulinic aciduria is due to inhibition of δ-aminolevulinate dehydratase by lead. Lead also inhibits purified ferrochelatase; however, the magnitude of inhibition was essentially nil even under pathological conditions. Further study proved the disturbance of iron-reducing activity by moderate lead exposure. Far different from these two enzymes, lead failed to inhibit purified coproporphyrinogen oxidase, i.e., the mechanism of coproporphyrinuria has not yet been understood. During the 80s to the 90s, the effects of environmental hazards including lead were elucidated through stress proteins, indicating the induction of some heme pathway enzymes as stress proteins. At that time, gene environment interaction was another focus of toxicology, since gene carriers of porphyrias are considered to be a high-risk group to chemical pollutants. Toxicological studies from the 70s to the 90s focused on the direct effect of hazards on biological molecules, such as the heme pathway enzymes, and many environmental pollutants were proved to affect cytosolic heme. Recently, we demonstrated the mechanism of the heme-controlled transcription system, which suggests that the indirect effects of environmental hazards are also important for elucidating toxicity, i.e., the hazards can affect cell functions through such biological mediators as regulatory heme. It is, therefore, probable that toxicology in the future will focus on biological systems such as gene regulation and signal transduction systems. ———— heme metabolism; lead; δ-aminolevulinate dehydratase; δ-aminolevulinate synthase; Bach1

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Lead intoxication and porphyria

It is well known that lead is one of the most famous inducers of chemical porphyria (National Academy of Sciences 1972). As typical signs of lead poisoning, δ-aminolevulinic aciduria, coproporphyrinuria, and accumulation of free- and zinc-protoporphyrin in erythrocytes were well described in the 1950s. According to these signs, the inhibition of three heme pathway enzymes, namely, δ-aminolevulinate (ALA) dehydratase (Koike 1959; Lichtman and Feldman 1963), coproporphyrinogen oxidase, and ferrochelatase, was suggested (Fig. 1). The biochemical mechanisms of these lead poisoning signs were elucidated in the 1970s, concurrent with the purification and characterization studies of these enzymes (Tsukamoto et al. 1979;

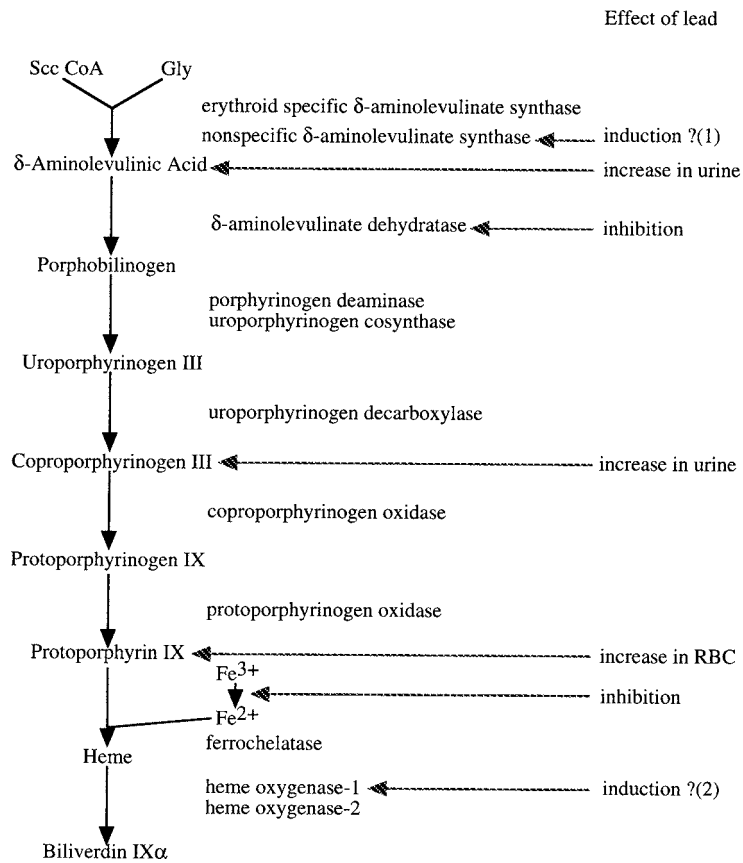

Fig. 1. Effect of lead on heme metabolism.

ALA dehydratase is a zinc-dependent enzyme, and the addition of lead in vitro competitively decreases zinc molecules in the enzyme. According to the loss of zinc in ALA dehydratase due to exchange with lead, the enzyme activity is inhibited in a dose-dependent manner (Tsukamoto et al. 1979).

Fifty percent inhibition of ferrochelatase and iron reducing activities in vitro was observed in the presence of 100 mM and 10 mM lead, respectively (Taketani and Tokunaga 1981; Taketani et al. 1985). Although immunoprecipitation abolished not only Fe chelating activity but also Zn chelating activity of ferrochelatase, a higher level of lead inhibited only Fe chelating activity of the enzyme. It is, therefore, probable that inhibition of ferrochelatase activity is absent in lead poisoning, resulting in the increase in Zn protoporphyrin in erythrocytes due to the decrease in Fe^{2+} supply.

(1) Induction of ALA dehydratase was described by Inoue (1959) and Maxwell and Meyer (1976).

(2) Induction of heme oxygenase was reported by Maines and Kappas (1976).

Yoshinaga and Sano 1980; Taketani and Tokunaga 1981). It should be noted that Japanese toxicologists pioneered these enzymatic studies on heme biosynthesis.

The biochemical mechanism of ALA dehydratase inhibition by lead was elucidated by Tsukamoto et al. (1979). Lead binds to the essential SH residues of purified hepatic ALA dehydratase, resulting in the loss of binding affinity to the substrate, δ-aminolevulinic acid (ALA). Since Millar et al. (1970) reported that inhibition of ALA dehydratase in liver was 60% of that in erythrocytes, the existence of tissue-specific isozyme has been suggested. The suggestion was, however, negated by enzymatical and immunochemical studies (Fujita et al. 1985, 1986), and tissue differences in ALA dehydratase inhibition were explained by differences in the relative concentrations among ALA dehydratase, lead, zinc and thiol in these tissues (Fujita et al. 1985). Lead also inhibits ferrochelatase activity in vitro; however, the magnitude of inhibition in vitro is less than that in vivo (Taketani and Tokunaga 1981). Taketani et al. (1985) demonstrated that the inhibition of ferrochelatase by lead in vivo is attributable to the decrease in iron-reducing activity. Ferrochelatase has zinc-chelating activity (Taketani and Tokunaga 1981) and ferrochelatase activity is not inhibited by moderate lead exposure, therefore, decreased supply of ferrous ion resulted in an increase in zinc protoporphyrin. Thus, the mechanisms of δ-aminolevulinic aciduria and accumulation of free- and zinc- protoporphyrin in erythrocytes are biochemically elucidated. In contrast, Yoshinaga and Sano (1979) reported no obvious inhibition of purified coproporphyrinogen oxidase in vitro. In the 1970s, the paradigm on studies of porphyria due to lead poisoning is, therefore, that on the biochemistry of heme metabolism itself.

Biological response against lead

Studies to clarify the enzymatic basis of inherited porphyrias were followed by the molecular analyses of mutations (Fujita et al. 1998). At this time, studies in lead-induced porphyrias should be different from those in inherited porphyrias. It is generally accepted that the elucidation of the reaction between a chemical and an enzyme will be the end of in vitro studies of chemical porphyrias. On the contrary, the enzymatic elucidation is the start of in vivo investigations, since humans have managed to survive in spite of the chemical exposure. Therefore, we thought that the enzymatic study of lead-induced porphyria in vitro must be succeeded by the investigation of the effects of lead on the enzyme synthesis in vivo (Fujita et al. 1981, 1982; Fujita and Ishihara 1988a).

As was expected, lead increased ALA dehydratase concentration as if to compensate the enzyme inhibition not only in rat but also in human (Fujita et al. 1981, 1982). When the onset of enzyme increase in peripheral red blood cells was compared with that in bone marrow cells, the latter was five to seven days earlier than the former, suggesting that the enzyme induction is attributable to the up-regulation of the gene by lead exposure (Fujita et al. 1981). A cell-free analysis supported the idea that lead induces the de novo synthesis of ALA dehydratase (Fujita and Ishihara 1988a).

Similar observations have been reported in the field of stress response. For example, heme oxygenase-1 gene, which encodes the key enzyme for heme degradation, is activated not only by its substrate, heme (Shibahara et al. 1987), but also by a series of stress, such as heat shock (Shibahara et al. 1987; Mitani et al. 1989, 1991), acute phase (Mitani et al. 1992), active oxygen (Tacchini et al. 1993; Maines et al. 1993), and heavy metals (Taketani et al. 1989; Mitani et al. 1993). Thus, research in lead poisoning in the 1980s to the 1990s was similar to that in mechanisms in response to stress.

Why mature erythrocytes contain a large amount of ALA dehydratase, in spite of the fact that mature erythrocytes no longer synthesize

heme for hemoglobin, has not been well understood. One of the possible roles of the enzyme protein in erythroid cells is as a 240 kDa lead-binding protein (Piomelli 1993), since it has been reported to contain 60 to 70% of blood lead (Bergdahl et al. 1997). This observation is in good agreement with a regression curve obtained between blood lead concentration (μM) as X and concentration of inhibited ALA dehydratase (subunit; μM) as Y in lead-exposed workers; $Y = -0.0547 + 1.448X - 0.3159X^2$, suggesting that 70% of lead binds to ALA dehydratase when the blood lead level is lower than 60 mg/100 ml (Fujita et al. 1982; Fujita 1999) (Fig. 2). Thus, the induction of ALA dehaydratase may decrease free lead to protect humans from the toxicity (Fujita et al. 1994).

Gene-environment interaction

Another focus of toxicological research is gene-environment interaction, since both genetic defect and intoxication by chemicals are the major etiologies of porphyrias (Sassa et al. 1987). ALA dehydratase defect porphyria (ADP) is so rare that only six families have been reported to date (Fujita et al. 1994, 1998; Akagi et al. 2000). It is well noted that ALA dehydratase activity in two major organs of heme biosynthesis, i.e., liver as well as bone marrow, is almost 100 times higher than the activity of ALA synthase, the rate-limiting step. In fact, ALA dehydratase activity in patients with ADP ranges from $<2\%$ to 12% of that in control (Fujita et al. 1994). Thus, only the homozygous defect develops signs and symptoms of the porphyria (Fujita et al. 1994, 1999).

In contrast to the rare incidence of ADP, as much as 2% of "normal" humans have less than 50% of the normal ALA dehydratase activity (Thunell et al. 1987). At least a part of this decreased ALA dehydratase activity should be

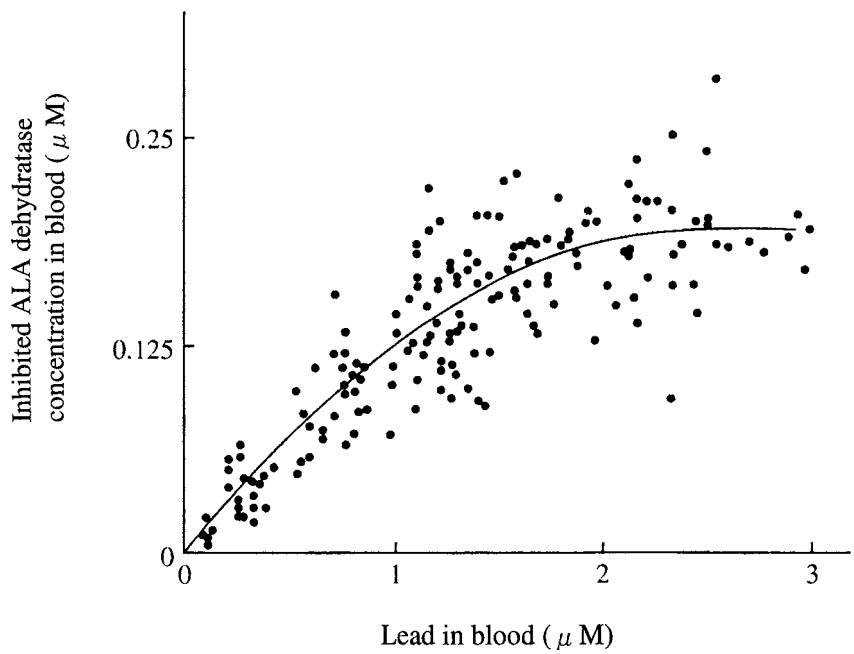

Fig. 2. Relationship between lead in blood and inhibited erythrocyte ALA dehydratase. ALA concentrations were estimated in lead-exposed workers, and inhibited enzyme concentration in each specimen was calculated from the inhibition rate of the enzyme (Fujita et al. 1982). Since one molecule of ALA dehydratase consists of eight identical subunits, 0.125 μM of the enzyme is equivalent to 1 μM ALA dehydratase subunit. The regression curve suggests that one molecule of lead is bound to one molecule of the enzyme subunit, when blood lead concentration is lower than 1.5 μM.

attributed to the heterozygous defect of the enzyme. These heterozygotes with ALA dehydratase deficiency may have a higher risk when exposed to environmental hazards (Fujita et al. 1994). Our study demonstrated that not only lead, but also trichloroethylene (Fujita et al. 1984; Koizumi et al. 1984a), bromobenzene (Koizumi et al. 1984b; Fujita and Ishihara 1988b) and styrene decreases ALA dehydratase activity (Fujita et al. 1986, 1987). Among these chemical hazards, lead and trichloroethylene inhibit ALA dehydratase activity via chemical modification of the active site (Tsukamoto et al. 1979; Fujita et al. 1984). Bromobenzene and styrene, however, inhibit the synthesis of ALA dehydratase, resulting in a marked reduction of the enzyme concentration (Fujita et al. 1986, 1987; Fujita and Ishihara 1988b). The incidence of subclinical exposures to these chemical hazards is of concern not only in industrial settings but also among the general population; therefore, the detection of gene carriers of ALA dehydratase defect may be important in genetic counseling for exposure to toxic substances.

Environmental stress and heme metabolism

Studies in the preceding section indicated that some of the environmental hazards inhibit heme biosynthesis. Recently, a tiny island in Kagawa Prefecture named Teshima became famous due to the illegally scrapped industrial wastes of up to 500 000 tons. In addition to the chemicals described here, dioxin, cadmium, and arsenite were detected in the waste. These chemical hazards are known to induce heme oxygenase-1, the key enzyme for heme catabolism (Taketani et al. 1989; Mitani et al. 1993). It is probable that the inhibition of heme biosynthesis as well as the up-regulation of heme degradation decreases heme concentration in cells; however, the significance of reduced heme level remains not well understood.

In the liver of trichloroethylene-exposed rats (Fujita et al. 1984) (Fig. 3), the inhibition of ALA dehydratase reduced cytochrome P-450 concentration and heme saturation of tryptophan pyrrolase, both of which are known to be indicators of free (or regulatory) heme pool. When we examined hepatic ALA synthase, the first step of heme biosynthesis, in treated animals, a marked induction was observed in a dose-dependent manner. Since a part of heme is considered to control ALA synthase expression for negative feedback regulation of heme biosynthesis, the observed induction of hepatic ALA synthase suggests the role of free heme (Yamamoto et al. 1988; Fujita et al. 1991a). Further analysis revealed that the gene of hepatic ALA synthase is located in 3p21.1.

An antibody against hepatic ALA synthase could not recognize ALA synthase in erythrocytes, suggesting that ALA synthase in erythrocytes and that in hepatic cells are isozymes (Yamamoto et al. 1986). The gene of ALA synthase in erythrocytes was found at Xp11.21; therefore, the enzyme was proven to have two isozymes: erythroid-specific ALA synthase and nonspecific ALA synthase (Fujita et al. 1991b). In murine Friend virus-transformed erythroleukemia (MEL) cells under hemin treatment, mRNA for erythroid-specific ALA synthase is markedly increased, suggesting an up-regulation of the gene (Fujita et al. 1991a). The induction of erythroid-specific ALA synthase by heme is physiologically important, since a large amount of heme for hemoglobin is essential in cells undergoing erythroid differentiation.

The gene encoding heme oxygenase-1, the inducible isozyme of heme oxygenase, is well known to be activated by heme, the substrate (Shibahara et al. 1987; Yoshida et al. 1988). It is also indicated that heme activated globin gene (Fukuda et al. 1994; Harigae et al. 1998). Thus, regulatory heme is suggested to control genes related to heme and hemoglobin metabolism; however, the exact mechanism for activating genes has not yet been elucidated.

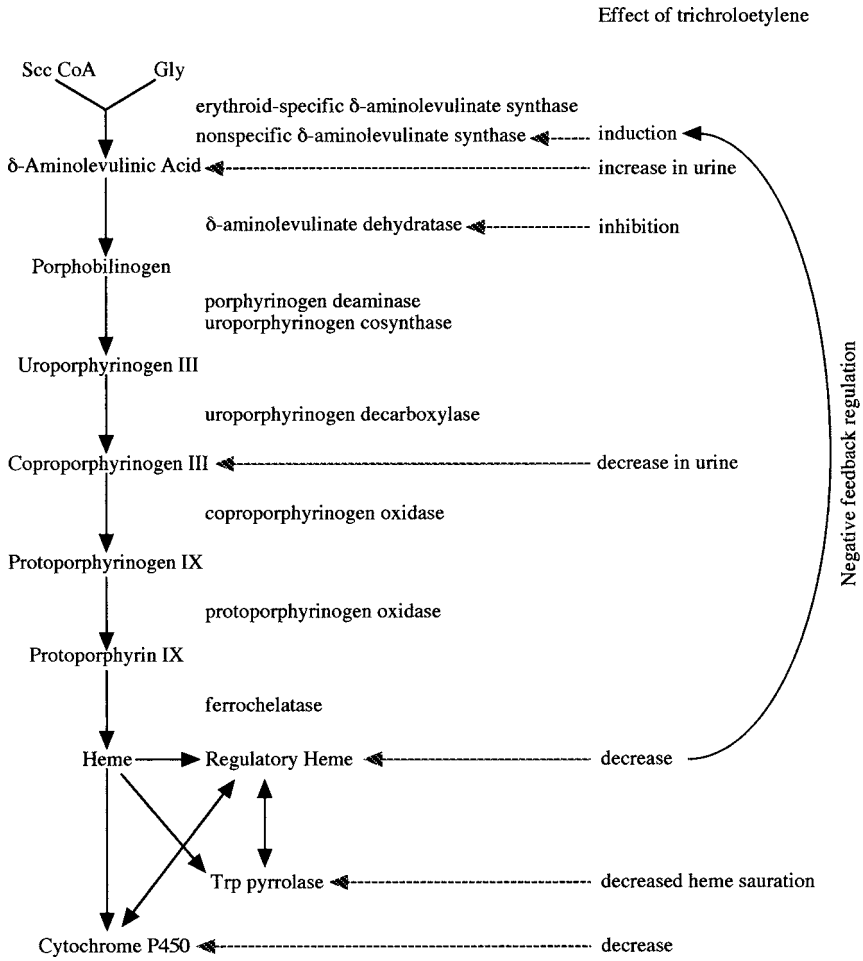

Fig. 3. Effect of trichloroethylene on heme biosynthesis.
Trichloroethylene is oxidized by cytochrome P450 to form reactive intermediate(s), and affects cytochrome P450 concentration through accelerated degradation. Since ALA dehydratase is an SH-dependent and SH-rich (8 Cys/subunit) enzyme, the intermediate(s) reacts with essential SH residues to cause irreversible inhibition.

Mechanism of heme-mediated control of genes

Maf recognition elements (MAREs) are present in the regulatory region of various genes involved in heme metabolism, such as oxidative stress-responsive genes (Ishii et al. 2000), globin genes (Talbot and Grosveld 1991; Kotkow and Orkin 1995; Igarashi et al. 1998; Yoshida et al. 1999), the heme oxygenase-1 gene (Inamdar et al. 1996; Alam et al. 1999), and the erythroid-specific ALA synthase gene (Kramer et al. 2000). Heterodimers of Maf-related oncoprotein family with transcription factor NF-E2 and its related factors Nrf1, −2 and −3 (Oyake et al. 1996; Marini et al. 1997; Johnsen et al. 1998; Kobayashi et al. 1999) bind to MAREs (Igarashi et al. 1994, 1995; Kataoka et al. 1995; Motohashi et al. 1997; Toki et al. 1997). Like NF-E2 family proteins, transcription factors Bach1 and Bach2, which are basic leucine zipper (bZip) proteins, form heterodimers with the Maf proteins to bind MAREs, functioning primarily as repressors (Oyake et al. 1996; Igarashi et al. 1998; Muto et al. 1998; Yoshida et al. 1999; Kobayashi et al. 2000). Thus, the gene regulation operating through MAREs might be based on the balance between activation and repression (Fig. 4).

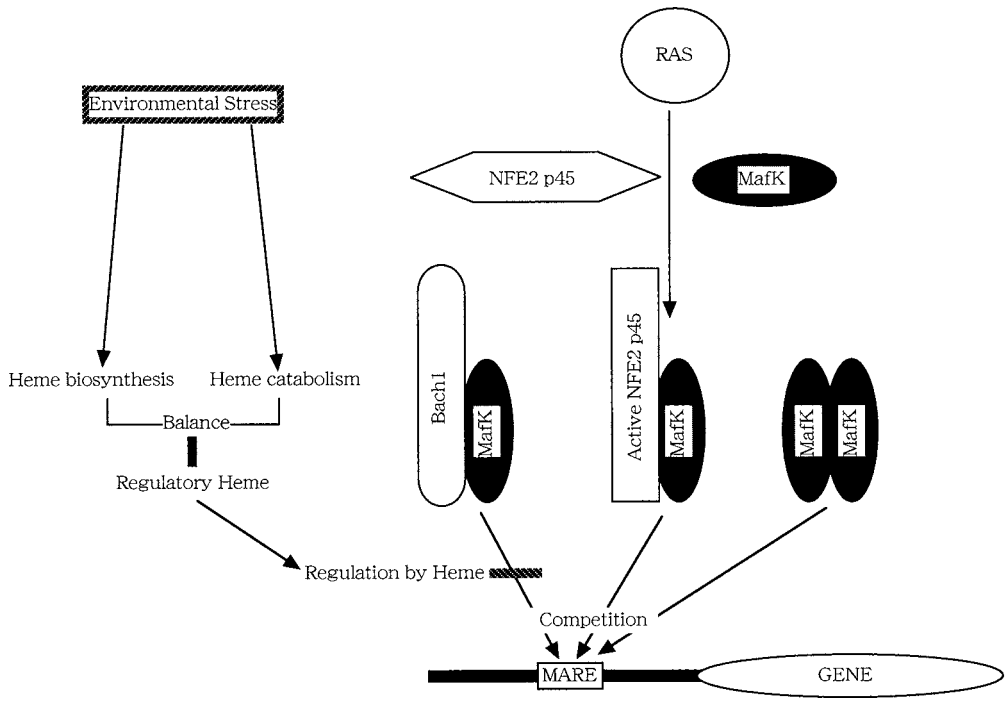

Fig. 4. Scheme of heme-controlled genes.
Effects of environmental stress on heme metabolism regulate genes through the DNA binding activity of the Ba1/MafK heterodimer.

To examine if Bach1 is a heme-binding protein, we expressed various portions of Bach1 as fusion proteins with glutathione S-transferase (GST) and carried out binding assays (Ogawa et al. 2001). Bach1 has at least two functional domains, i.e., a BTB/POZ domain involving protein interaction in the N-terminal region and a DNA- binding bZip domain in the C-terminal region. When a portion of Bach1 without the BTB/POZ domain (amino acids 174–739) was incubated with hemin, a Soret band with a peak at 421 nm was observed, indicating that Bach1 is a heme-binding protein. Scatchard analysis revealed that 1.3 mol of hemin bound to 1 mol of the portion of Bach1 (amino acids 174–739) with a Kd of 140 nM.

Using several GST fusions of Bach1 derivatives (portions of amino acids 174–739, 174–415, 417–739, 417–645, and 558–739), the heme-binding region was explored. Although the N-terminal portion (amino acids 174–415) showed no specific binding to heme, the C-terminal portion (amino acids 417–739) showed a high affinity for heme that was as high as a portion of Bach1 without the BTB/POZ domain (amino acids 174–739). Two subfragments (417–645 and 558–739) still showed comparable heme-binding activities that were almost half of that of the C-terminal portion (amino acids 417–739). In the C-terminal portion (amino acids 417–739), there are four Cys and Pro dipeptide (CP) motifs, which have been found in heme regulatory motifs (HRMs) to play an important role in heme binding (Creusot et al. 1989; Chen et al. 1991; Rotenberg and Maines 1991; Lathrop and Timko 1993; Steiner et al. 1996; McCoubrey et al. 1997). When we examined GST fusion proteins with single amino acid substitutions of each CP motif by changing Cys to Ala, no decline in heme-binding activity was observed. In contrast, replacement of three clustered CPs upstream of the bZip domain or of all four CPs reduced heme binding activity to 1/3 or to 1/10 of the control, respectively. Thus,

heme binding of Bach1 is mainly regulated by four CP motifs in the C-terminal portion (amino acids 417–739).

The specific binding of heme by CP motifs suggests that heme in Bach1 has certain functions; therefore, the effects of heme on MARE binding activity were analyzed. Addition of hemin to the DNA binding reactions showed a dose-dependent inhibition of binding activity by Bach1-MafK heterodimer. Even 0.03 μM heme caused reproducible inhibition, and almost complete inhibition was observed in the presence of 1 μM heme. Cys to Ala substitution of four CP motifs in Bach1 abolished heme-mediated inhibition of DNA binding. It is, therefore, suggested that the four CP motifs play an essential role in the regulatory function of heme via Bach1 binding. There are two possible mechanisms for reducing DNA binding by heme: one is the direct inhibition of Bach1-MafK heterodimer binding to MARE, and the other is through reduced heterodimer formation. The latter possibility was immunochemically examined. Addition of 1 μM hemin had no effect on leucine zipper-mediated formation of Bach1-MafK, suggesting that heme inhibits the DNA binding activity of the heterodimer.

The functional effects of the interaction between heme and Bach1 in cells were examined using a MARE-dependent reporter plasmid. Without hemin treatment, Bach1 repressed the expression of the reporter gene, while 10 μM hemin in the medium abolished the repression by Bach1. When all CPs were altered to APs, Bach1 repressed the gene expression even in the presence of 10 μM hemin. It is, therefore, proved that one of the mechanisms of heme-controlled transcription is the regulation of repression activity of Bach1 interacting with CP motifs.

In cells with low heme concentration, Bach1 represses genes with MAREs in their regulatory regions. When cellular heme increases, heme reduces the DNA-binding activity of Bach1, resulting in the dissociation of Bach1 from the enhancers. A higher level of heme, therefore, opens MAREs to competing activators such as Nrf2 (Alam et al. 1999; Ishii et al. 2000) and NF–E2 p45 (Igarashi et al. 1998). We have reported that NF-E2 p45-MafK heterodimer binding activity is markedly induced in erythroid-differentiating MEL cells (Nagai et al. 1998). Since there is significant induction of heme biosynthesis during erythroid differentiation (Fujita et al. 1991b), cellular heme level may decrease DNA binding activity of Bach1 to increase NF-E2-DNA complex. No increase in NF-E2-DNA complex was observed in DR clone, a clone of MEL cell that fails to undergo differentiation due to absence of erythroid-specific ALA synthase (Fujita et al. 1991a; Nagai et al. 1998). One of the etiologies of sideroblastic anemia, a heterogeneous disorder of erythroid differentiation, is insufficient heme biosynthesis and a part of sideroblastic anemia may be related to neoplasia, i.e., a clonal disorder that can progress to leukemia (Beutler 1995). Since we found that the function of NF-E2 was under the control of the Ras-Raf-MAP kinase signaling cascade (Nagai et al. 1998), one of the functions of the Bach1/heme system seems to be as a biological modulator of the signal transduction. Thus, the heme-controlled function of Bach1 seems to be one of the essential factors in transcription as well as in signaling for normal cell differentiation.

Many of the environmental hazards described herein affect cellular heme concentration either by inhibition of heme biosynthesis or by induction of heme degradation. It is possible that the affected cellular heme, a biological modulator, by some environmental pollutants disturbs normal cellular functions, including differentiation, as described above. Further studies are necessary to examine this possibility. Nevertheless, our observations demonstrated that the indirect effects, such as via the heme/Bach1/NF-E2 system, of chemical hazards must be elucidated in the near future in the field in toxicology.

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