Bach1 Deficiency Ameliorates Hepatic Injury in a Mouse Model

AKIO IIDA,1 KOJI INAGAKI,1 AKIRA MIYAZAKI,1 FUMIHIKO YONEMORI,1 ETSURO ITO2 and KAZUHIKO IGARASHI3

1Japan Tobacco Inc., Central Pharmaceutical Research Institute, Osaka, Japan
2Department of Pediatrics, Hirosaki University School of Medicine, Hirosaki, Aomori, Japan
3Department of Biochemistry, Tohoku University Graduate School of Medicine, Sendai Japan

Bach1 is a basic region-leucine zipper (bZip) protein that forms heterodimers with the small Maf proteins and functions as a repressor of gene expression. One of the target genes of Bach1 is Hmox-1 that encodes heme oxygenase-1 (HO-1). HO-1 degrades heme into carbon monoxide (CO), biliverdin, and iron. HO-1 is strongly induced by various stresses as well as its substrate heme, and protects cells and tissues against insults through diverse cytoprotective functions of the reaction products CO and biliverdin. Bach1-deficiency in mice leads to higher expression of Hmox-1 in various tissues. Here we investigated the effects of Bach1-deficiency in mice on tissue injuries: hepatic injury induced by D-galactosamine (GalN) and lipopolysaccharide (LPS), and mouse paw edema induced by carrageenan, polysaccharide derived from various seaweeds. Bach1-deficiency suppressed induction of plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in response to the GalN/LPS-treatment. However, production of tumor necrosis factor alpha (TNF-α) and nitric oxide (NO), both being cytotoxic mediators in LPS-induced hepatic injury, in Bach1-deficient mice and their peritoneal macrophages was similar to wild type controls. In contrast, Bach1-deficiency did not affect extent of mouse paw edema induced by carrageenan, which enhances vascular permeability by activating kinin release. These results indicate that Bach1 plays an inhibitory role in the cytoprotection of LPS-induced liver injury but not in the kinin-mediated inflammatory edema. The inhibitory role for Bach1 may stem from its activity to repress gene expression including HO-1.

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Bach1 is a basic region-leucine zipper (bZip) transcription factor that forms heterodimers with the small Maf proteins. The resulting Bach1 heterodimers bind to the Maf recognition elements (MAREs) to repress transcription (Oyake et al. 1996; Igarashi et al. 1998; Ogawa et al. 2001; Sun et al. 2002; Sun et al. 2004). Other bZip transcription factors such as NF-E2 related factor (Nrf)1 and Nrf2, distantly related to Bach1 (Amoutzias et al. 2007), also form heterodimers with the small Maf proteins, bind to MARE, and activate transcription. As such, MARE-dependent transcription is finely tuned by both repressors and activators (Motohashi et al. 2002; Igarashi and Sun 2006). The balance of repression and activation is modulated in part by heme: direct binding of heme to Bach1 inhibits the DNA binding activity of Bach1/small Maf heterodimer and induces nuclear export of Bach1 (Ogawa et al. 2001; Sun et al. 2002; Suzuki et al. 2004). Furthermore, heme induces polyubiquitination and subsequent degradation of Bach1 (Zenke-Kawasaki et al. 2007). MARE or MARE-like sequences are present in regulatory regions of various genes that are related to heme, oxidative stress response, and xenobiotics metabolism (Kyo et al. 2004). One of the well characterized target genes of Bach1 is Hmox-1 that encodes heme oxygenase-1 (HO-1). Expression of Hmox-1 in cultured cells or in organs is very low under normal conditions due to Bach1-mediated repression but is highly induced by its substrate heme, oxidative stress, or other diverse stimuli such as cytokines, heavy metals and heat shock (Shibahara et al. 1985, 1987; Alam et al. 1989; Keyse and Tyrrell 1989; Taketani et al. 1989). Hmox-1 is constitutively expressed at higher levels in many tissues of Bach1-deficient mice, indicating that Bach1 acts as a negative regulator of transcription of Hmox-1 (Sun et al. 2002; Omura et al. 2005).

HO-1 catalyzes oxidative degradation of heme, generating iron, carbon monoxide, and biliverdin, which is rapidly reduced to bilirubin. Carbon monoxide, biliverdin, and bilirubin possess antioxidant and anti-inflammatory actions (Baranano et al. 2002; Otterbein et al. 2003). Consistent with their protective actions, induction of HO-1 under diverse stress conditions have been shown to protect cells and tissues. For example, HO-1 protects cultured fibroblasts and endothelial cells from TNF-induced apoptosis.


Inhibitory role of Bach1 in cytoprotection α and/or other genes. Consistent with this idea, -deficiency (Yachie et al. 1999). α gene has been deficiency on galactosamine (GalN)/- - 225 -α-225 λ No. 6, March 27, 1980, of the Prime Minister's Office of Japan).

To further understand the function of Bach1 in protective responses against various stresses, we examined the effects of Bach1 deficiency on galactosamine (GalN)/lipopolysaccharide (LPS)-induced liver injury, which is known to be alleviated by induction of HO-1 (Sass et al. 2003). It was reported previously that LPS-induced hepatic injury is caused by hepatic apoptosis mediated by tumor necrosis factor alpha (TNF-α) and nitric oxide (NO) (Lehmann et al. 1987; Morikawa et al. 1999; Sass et al. 2003; Wolf et al. 2005). Thus, we compared LPS-induced TNF-α and NO production in Bach1-deficient and control mice. Because HO-1 suppresses inflammatory response to carrageenin (Willis et al. 1996), we also examined the effect of Bach1-deficiency in carrageenin-induced mouse paw edema as an inflammation model.

**Materials and Methods**

**Reagents**

Galactosamine (GalN) was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Lipopolysaccharide (LPS) was from Sigma (St Louis, MO). Hyperfilm ECL and ECL plus detection reagent were from Amersham Biosciences, Inc. (Buckinghamshire, England). Horseradish peroxidase-coupled goat anti-rabbit IgG was from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, Maryland). λ-Carrageenin (Picnin A) was from Zushikagaku Laboratory (Kanagawa, Japan).

**Animals**

Generation of Bach1-/- mice on a C57BL/6J background has been reported elsewhere (Sun et al. 2002). Bach1+/+, Bach1-/- and Bach1+/ mice aged 7–12 weeks were kept on a 12-h light / dark cycle with free access to food and sterile water. All the experiments received prior approval from the committee for the human care and use of animals of our laboratory (Japan Tobacco Inc., Central Pharmaceutical Research Institute), in accordance with the Standards Relating to the Care and Management of Experimental Animals (Notification No. 6, March 27, 1980, of the Prime Minister’s Office of Japan).

GalN/LPS-induced liver injury model

Mice were injected intraperitoneally (i.p.) with GalN (500 mg/kg) and LPS (3 μg/kg). Heparinized blood was obtained from the orbital sinus of the mice at 1 and 6 hours after GalN/LPS injection. Blood plasma was separated from heparinized whole blood by centrifugation at 2000 × g for 3 minutes at 4°C. The activities of plasma ALT and AST at 6 hours after GalN/LPS injection, the marker enzymes of liver injury, were measured with Monarch (Instrumentation Laboratory, Lexington MA). Plasma concentration of TNF-α at 1 hour after GalN/LPS injection was measured with commercial murine TNF-α ELISA kit (Quantikine M; R&D Systems, Mineapolis, MN).

**Western Blot Analysis**

Insoluble materials in the lysis samples described above were removed by centrifugation at 15,000 × g at 4°C for 10 minutes. The supernatants (1 mg protein/ml) were boiled in SDS-PAGE sample buffer for 3 minutes. After a brief centrifugation, the supernatants (20 μl/lane) were separated by SDS-PAGE and then electroblotted onto nitrocellulose membrane. The blots were blocked with 3% skim milk in PBS at room temperature for 1 hour and then incubated with primary anti-HO-1 antibody (Stressgen) at 1 : 2000 at room temperature for 1 hour. After washing with PBS containing 0.1% Tween 20, the blots were probed with secondary antibody (horseradish peroxidase-coupled goat anti-rabbit IgG at 1 : 5000) at room temperature for 1 hour. After a second wash, the blots were exposed to Hyperfilm ECL and ECL plus detection reagent to enable visualization of phosphoryrosine-containing proteins.

Carrageenin-Induced Paw Edema

Right hind paw of mouse was measured by plethysmometer (TK-101; Unicom Inc., Chiba, Japan) 2 h before Carrageenin inoculation. Carrageenin was dissolved in saline by incubation for 24 h at
4°C to make up 1% solution and inoculated subcutaneously at a volume of 50 μl at foot pad of the right hind paw. At 2, 3, 5, 24 hours after carrageenin injection, paw volume was measured and compared with predosing value, and the edema formation of each mouse was determined. All the measurements were performed in a blind manner. Data were calculated as percentage of increase of the paw volume by comparing pre- and post-carrageenin injection.

Statistical analysis

Data are expressed as mean ± SEM. The statistical significance was determined by Student’s t test or Aspin-welch t test.

RESULTS

Effects of Bach1-deficiency on GalN/LPS-induced liver injury

Combined injection of GalN and LPS causes massive liver injury due to apoptotic cell death (Morikawa et al. 1996). We compared the effects of Bach1-deficiency on GalN/LPS-induced liver injury using Bach1+/+, Bach1+/- and Bach1−/− mice. Injection of GalN/LPS significantly increased plasma ALT and AST activities in Bach1+/+ mice that reflected liver injury (Fig. 1A and B). Release of ALT and AST activities after GalN/LPS treatment was significantly suppressed in Bach1−/− mice. Interestingly, it was also reduced to some extent in Bach1+/- mice. Considering that LPS-induced cytotoxicity is mainly mediated by TNF-α (Lehman et al. 1987; Tiegs et al. 1989; Morikawa et al. 1996), we next examined plasma levels of TNF-α (Fig. 1C). While the level of TNF-α in wild-type mice sera was 60-70 pg/ml without any treatment, it increased at 1 hour after GalN/LPS injection and then returned to basal level by 6 hours (data not shown). Plasma concentration of TNF-α in Bach1−/− mice similarly increased after injection of GalN/LPS. No significant change in viability of mice was observed.

Bach1-deficiency does not affect LPS-induced TNF-α and NO production in macrophages

Macrophages produce proinflammatory cytokines including TNF-α and IL-6 in response to LPS. To investi-

![Fig. 1. Effects of GalN/LPS injection on plasma AST, ALT and TNF-α levels in control and Bach1+− mice. (A and B) The activities of plasma aminotransferase (ALT and AST) at 6 hours after GalN/LPS injection. Each value represents mean ± SEM ([GalN/LPS(-)] Bach1+/+, n = 8; Bach1−/−, n = 9; [GalN/LPS(+)] Bach1+/+, n = 5; Bach1+/-, n = 5; Bach1−/−, n = 6). (C) Plasma concentration of TNF-α at 1 hour after GalN/LPS injection. Each value represents mean ± SEM (Bach1+/+, n = 5; Bach1−/−, n = 5; Bach1+/-, n = 6). Statistical analysis was performed by Student’s t test (**, p < 0.01) or by Aspin-welch t test (#, p < 0.05) v.s. control mice.]
Inhibitory role of Bach1 in cytoprotection

To gate the mechanisms of reduction of plasma ALT and AST by Bach1-deficiency in GalN/LPS-induced liver injury, we assessed the role of Bach1 in the response to LPS in vitro using peritoneal macrophages isolated from Bach1+/+ and Bach1−/− mice. Stimulation with LPS markedly increased TNF-α secretion from macrophages from Bach1+/+ and Bach1−/− mice without any significant difference (Fig. 2).

LPS is known to induce NO production in macrophages (Lee et al. 2004). It was suggested previously that NO may participate in the development of liver injury (Morikawa et al. 1999). To examine whether Bach1 is involved in the production of NO in macrophages, isolated macrophages were stimulated with or without LPS, and levels of NO were determined. The basal levels of NO production were comparable in control and Bach1+/+ macrophages (Fig. 3). When stimulated with LPS, NO production was strongly induced in both macrophages, with no significant difference (Fig. 3). Since there is no report regarding HO-1 expression in Bach1-deficient macrophages, we examined LPS-induced HO-1 expression in peritoneal macrophages from Bach1+/+ and control mice. Stimulation with LPS ex vivo increased HO-1 expression in Bach1+/+ macrophages (Fig. 4). In contrast, HO-1 expression in macrophages from Bach1−/− mice was much higher than those from Bach1+/+ mice even without LPS stimulation. Thus, it remains possible that overexpressed HO-1 modulates functions of Bach1-deficient macrophages other than the production of TNF-α and NO.

Effect of Bach1-deficiency on carrageenin - induced paw edema

As is the case of GalN/LPS-induced liver injury model,
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The increased HO-1 expression caused by Bach1 ablation may involve HO-1 overexpression in these cells. The increased HO-1 expression caused by Bach1-deficiency is expected to lead to degradation of heme and generation of CO, ferrous iron, and biliverdin, which is rapidly reduced to bilirubin. CO is associated with inhibition of cell injury and vascular dilatation (Otterbein et al. 2003; Sass et al. 2003) and bilirubin exerts protection against oxidative cellular damage (Clark et al. 2000). While heme induces increased vascular permeability, adhesion molecule expression, and leukocyte recruitment, HO-1 antagonizes the heme-induced inflammation (Ballal et al. 1991; Wagener et al. 2001). When taken together, it is conceivable that the protective effects caused by Bach1-deficiency in GalN/LPS-induced liver injury model reflect a composite mode of Bach1 action in hepatic cells and other cells including macrophages. It will be important to elucidate this complex network of cytoprotective responses at multiple levels.

Our previous (Omura et al. 2005; Yano et al. 2006) and current observations suggest that Bach1 inhibits cell and tissue survival in response to diverse stresses. Obvious enigma is whether Bach1 plays a role that allows natural selection instead as natural selection usually favors a system for survival. Considering that heme is involved in a variety of biological events by modulating the function or the state of heme proteins (Furuyama et al. 2007), the primary function of Bach1 may reside in the regulation of heme homeostasis. Bach1 may also play beneficial roles as well in stress responses, which may have eluded our analysis thus far.

In contrast to the GalN/LPS-induced liver injury model, there was no protective effect of Bach1-deficiency in carrageenin-induced paw edema. Carrageenin, a water-extractable polysaccharide obtained from various seaweeds, induces macrophage accumulation in fluid exudates from subcutaneous chambers in dogs (Hou et al. 2004). Carrageenin activates kinin release and induces kinin B1 receptor (Campos et al. 1996; Decarie et al. 1996; Ni et al. 2003). Bradykinin, one of kinins, enhances vascular permeability and then causes inflammatory edema. It also activates phospholipase A2 (PLA2), enhancing PGE2 production which then enhances vascular permeability and vasodilation. Thrombin can act as an inflammatory mediator in this model (Cirino et al. 1996). Bach1 may not be related to these inflammatory mediators and to edematous response.

In conclusion, the results of this study suggest that Bach1 regulates the response of hepatic cells to inflammatory stresses. Taken together with its augmenting role in ischemic reperfusion injury of heart and atherosclerosis (Omura et al. 2005; Yano et al. 2006), inhibition of Bach1 may provide new therapeutic approaches toward various diseases.

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References


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