Original Article

Bach1 gene ablation reduces steatohepatitis in mouse MCD diet model

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Bach1 is a transcriptional repressor of heme oxygenase-1 (HO-1, a.k.a. HSP-32), which is an inducible enzyme and has anti-oxidation/anti-inflammatory properties shown in various models of organ injuries. Since oxidative stress plays a pivotal role in the pathogenesis of nonalcoholic steatohepatitis (NASH), HO-1 induction would be expected to prevent the development of NASH. In this study, we investigated the influence of Bach1 ablation in mice on the progression of NASH in methionine-choline deficient (MCD) diet model. Bach1 ablation resulted in significant induction of HO-1 mRNA and its activity in the liver. When fed MCD diet, Bach1$^{-/-}$ mice exhibited negligible hepatic steatosis compared to pronounced steatohepatitis in wild type mice with 6-fold increase in hepatic triglyceride content. Whereas feeding of MCD diet decreased mRNA expressions of peroxisome proliferator-activated receptor (PPAR) α and microsomal triglyceride transfer protein (MTP) in wild type mice, there were no change in Bach1$^{-/-}$ mice. In addition, hepatic concentration of malondialdehyde (MDA), a biomarker for oxidative stress as well as plasma alanine aminotransferase (ALT) was significantly lower in Bach1$^{-/-}$ mice. These findings suggest that Bach1 ablation exerts hepatoprotective effect against steatohepatitis presumably via HO-1 induction and may be a potential therapeutic target.

Key Words: **oxidative stress, steatohepatitis, nonalcoholic fatty liver disease, heme oxygenase-1, peroxisome proliferator-activated receptor α**

Nonalcoholic fatty liver disease (NAFLD) is the most major liver dysfunction in the world and its prevalence is increasing with epidemics of obesity, diabetes, and metabolic syndrome. NAFLD includes wide-ranging liver disorder from simple steatosis through steatohepatitis to cirrhosis and possibly hepatocellular carcinoma.[1,2] About one-third of NAFLD is regarded as nonalcoholic steatohepatitis (NASH) which shows activity of steatohepatitis with the potential for a lethal outcome. In the development of NASH, "two hit theory" is assumed to be required, with the first hit being lipid accumulation in hepatocytes increasing the sensitivity of the liver to the second hit such as oxidative stress.[3] In this setting, emerging animal studies have demonstrated the protective effects of antioxidant (e.g., vitamin E, 1-aminobenzotriazole, curcumin, N-acetylcysteine, angiotensin II type I receptor blockers) in experimental models of NASH.[4–7] In human, small clinical trials have revealed potential usefulness of vitamin E in the treatment of NASH.[8–10] Interestingly, administration of fermented green tea extract which exerts antioxidant activity reduced hepatic triglyceride content in addition to necro/inflammation and fibrosis in rat NASH model.[11] These suggest that oxidative stress plays a pivotal role in the pathogenesis of NASH and expected to be one of the promising targets in the treatment of this disease.

Heme oxygenase (HO) is the rate limiting enzyme in the catabolism of heme, to yield biliverdin, carbon monoxide (CO), and free iron. HO exhibits antioxidant properties derived from the elimination of prooxidant heme as well as from biological activities of its reaction products, CO, biliverdin, bilirubin, and iron.[12] In mammals three isoforms, designated as HO-1, HO-2 and HO-3 have been identified in the liver. Among these HO isoforms, HO-3 is considered as processed pseudogenes derived from HO-2 transcripts.[13] While HO-2 is constitutively expressed mainly in parenchymal cells, HO-1 is prominent in Kupffer cells and is markedly induced in hepatocytes in the response to various stimuli. Interestingly, it has been reported that HO-1 expression was significantly increased in the liver from NASH patients and the increase reflected the severity of the disease.[14] These suggest that HO-1 functions as a defense system against oxidative stress in the liver. In fact, accumulating evidences have demonstrated hepatoprotective effects of HO-1. The induction HO-1 by cobalt protoporrphyrin (CoPP) protects human hepatocytes from ethanol-induced cytotoxicity.[15] *Ginkgo biloba* extract, a naturally occurring HO-1 inducer has been proved to ameliorate ethanol-induced sustained damage and redox imbalance on rat liver.[16] In addition, curcumin which has a beneficial effect on NASH has been recently proved to be an HO-1 inducer.[5,17]

In addition to cytoprotective properties of HO-1, the regulatory mechanisms of this gene have been revealed. A heme-binding factor Bach1 functions as a transcriptional repressor of the gene encoding HO-1 (*Hmox1*). Bach1 forms complexes with small Maf proteins and competes against NF-E2 related factor (Nrf2), the major transcriptional activator of *Hmox1*, for binding at the Maf recognition elements (MAREs) of *Hmox1* enhancer. Consistently, mice lacking Bach1 exhibited constitutively high level of HO-1 expression in many tissues including the liver.[18] Further studies of Bach1$^{-/-}$ mice have revealed a hepatoprotective role of Bach1 ablation in LPS-induced liver injury.[19]

In the current study, we investigated the influence of Bach1 disruption in animal NASH model. Accompanied with marked up-regulation of HO-1 in the liver, we observed reduced hepatic steatosis in Bach1$^{-/-}$ mice with altered expressions of key genes that regulate hepatic lipid metabolism. Consistent with these findings, the absence of Bach1 also exhibited hepatoprotection confirmed by serum liver enzyme and hepatic malondialdehyde (MDA) concentration. Collectively, these data suggest Bach1 as a potential target in the treatment of NASH presumably via induction of HO-1.

Materials and Methods

Animals and experimental protocol. Male C57BL/6 mice

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Table 1. Primer used for quantitative real-time PCR

Gene	Forward	Reverse
HO-1	acatcgacagccccaccaagttcaa	ctgacgaagtgacgccatctgtgag
PPARα	tgcaaacttggacttgaacg	tgatgtcacagaacggcttc
MTP	catgtcagccatcctgtttg	ctcgcgataccacagactga
αSMA	tcctccctggagaagagctac	tataggtggtttcgtggatgc
TGF-β	tgcgcttgcagagattaaaa	ctgccgtacaactccagtga
GAPDH	agaacatcatccctgcatcc	ttgtcattgagagcaatgcc

HO-1, heme oxygenase-1; PPARα, peroxisome proliferator-activated receptor alpha; MTP, microsomal triglyceride transfer protein; αSMA, alpha smooth muscle actin; TGF-β, transforming growth factor beta; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

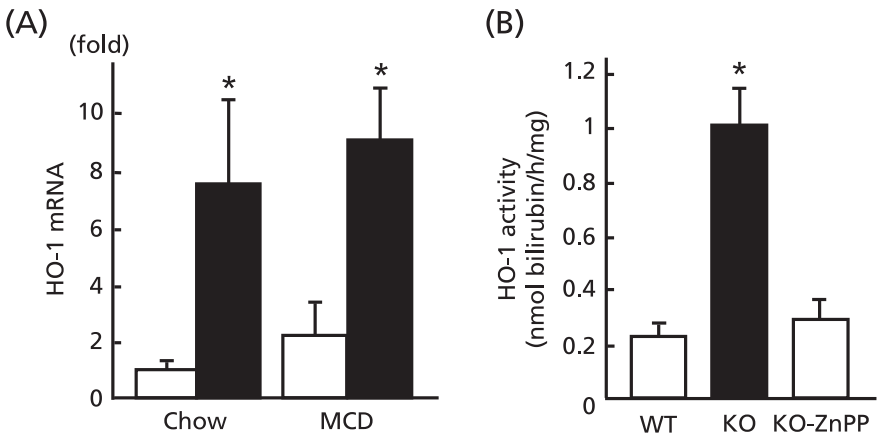

Fig. 1. Hepatic HO-1 expression and its activity in the absence of Bach1. (A) Wild type mice (open bar) and Bach1$^{-/-}$ mice (closed bar) were fed either regular chow or MCD diet for 8 w prior to quantification of hepatic expression of HO-1 mRNA by real-time PCR (n = 5/each group). (B) HO-1 activity in the liver from wild type (WT) or Bach1$^{-/-}$ (KO) mice were determined with standardlized of protein concentration (n = 5/each group). ZnPP was utilized as a HO-1 inhibitor for negative control. *p<0.05, wild type vs Bach1$^{-/-}$ mice.

were obtained from Charles River Laboratories (Yokohama, Japan). The generation of Bach1$^{-/-}$ mice on C57BL/6 background was described previously.[18] These mice at 10 weeks of age (5 mice per each group) were fed either methionine-choline deficient (MCD) diet (Oriental Yeast Co., Tokyo, Japan) or standard chow for 4 or 8 weeks with free access to drinking water. All animal protocols and studies were performed according to the guidelines of Institute of Laboratory Animal Science, Hiroshima University.

Histological studies. Liver samples were fixed in 10% formalin, embedded in paraffin, and sliced into 4 μm thick. These were subjected to standard procedure of hematoxylin and eosin (HE) staining or Azan-Mallory staining.

Biochemical assays. Serum alanine aminotransferase (ALT) levels were measured enzymatically. Hepatic triglyceride content was determined using Wako *E* test triglyceride kit (Wako chemical, Osaka, Japan) following lipid extraction.[20] Hepatic MDA levels were quantified using Lipid Peroxidation Assay Kit (Calbiochem, Gibbstown, NJ) with standardization of tissue protein concentrations.

HO activity. HO activity in the liver was determined as previously described.[21,22] In brief, microsome fraction isolated by ultracentrifugation was reacted with hemin as substrate, and bilirubin formation was measured using Beckman DU640 spectrophotometer (450 nm; Beckman Coulter, Fullerton, CA). The activity was expressed as nmol bilirubin formed per hour per mg of protein. To inhibit HO activity for a negative control, zinc protoporphyrin (ZnPP) in phosphate buffer was injected intraperitoneally at a dose of 7.5 mg/kg once a week for 8 weeks.

Quantitative real-time PCR. RNA was extracted from frozen liver tissues using RNeasy Mini Kit (Qiagen, Hilden, Germany). Complementary DNA was synthesized from 1 μg of total RNA with GeneAmp RNA PCR Core Kit (Applied Biosystems, Foster City, CA). Specific primers (Table 1) were designed using Primer 3 (http://frodo.wi.mit.edu/primer3/) on the basis of nucleotide sequences from Genbank. Quantitative real-time PCR was performed on Light Cycler system using Light Cycler FastStart DNA Master Plus SYBR Green I (Roche Applied Science, Basel, Switzerland). The mRNA expressions were normalized to the housekeeping gene, GAPDH as an internal control.

Statistical analysis. The results are expressed as the means ± SE. The statistical analysis was performed using Student's t test and differences were considered statistically significant when p was less than 0.05.

Results

Hepatic HO-1 expression and its activity in the absence of Bach1. Fig. 1 demonstrates the regulation of HO-1 expression and its activity by Bach1. As shown in Fig. 1A, real-time PCR quantified a 8-fold up-regulation of HO-1 mRNA in Bach1$^{-/-}$ mice on chow diet. Despite not being statistically significant, there was 2-fold up-regulation of mRNA by MCD diet in wild type mice. Consistent with these changes in mRNA expressions, Fig. 1B demonstrates 5-fold increase in hepatic HO-1 activity in the absence of Bach1, which was entirely repressed by intraperitoneal injection of ZnPP, an HO-1 inhibitor.

Influence of Bach1 ablation on hepatic lipid metabolism. Eight weeks feeding of MCD diet induced moderate infiltration of inflammatory cells with macrovesicular steatosis in the liver of wild type mice as depicted in Fig. 2A (A, B). In contrast to wild type mice, Bach1$^{-/-}$ mice appeared devoid of hepatic steatosis (C, D). In keeping with these morphological changes, hepatic

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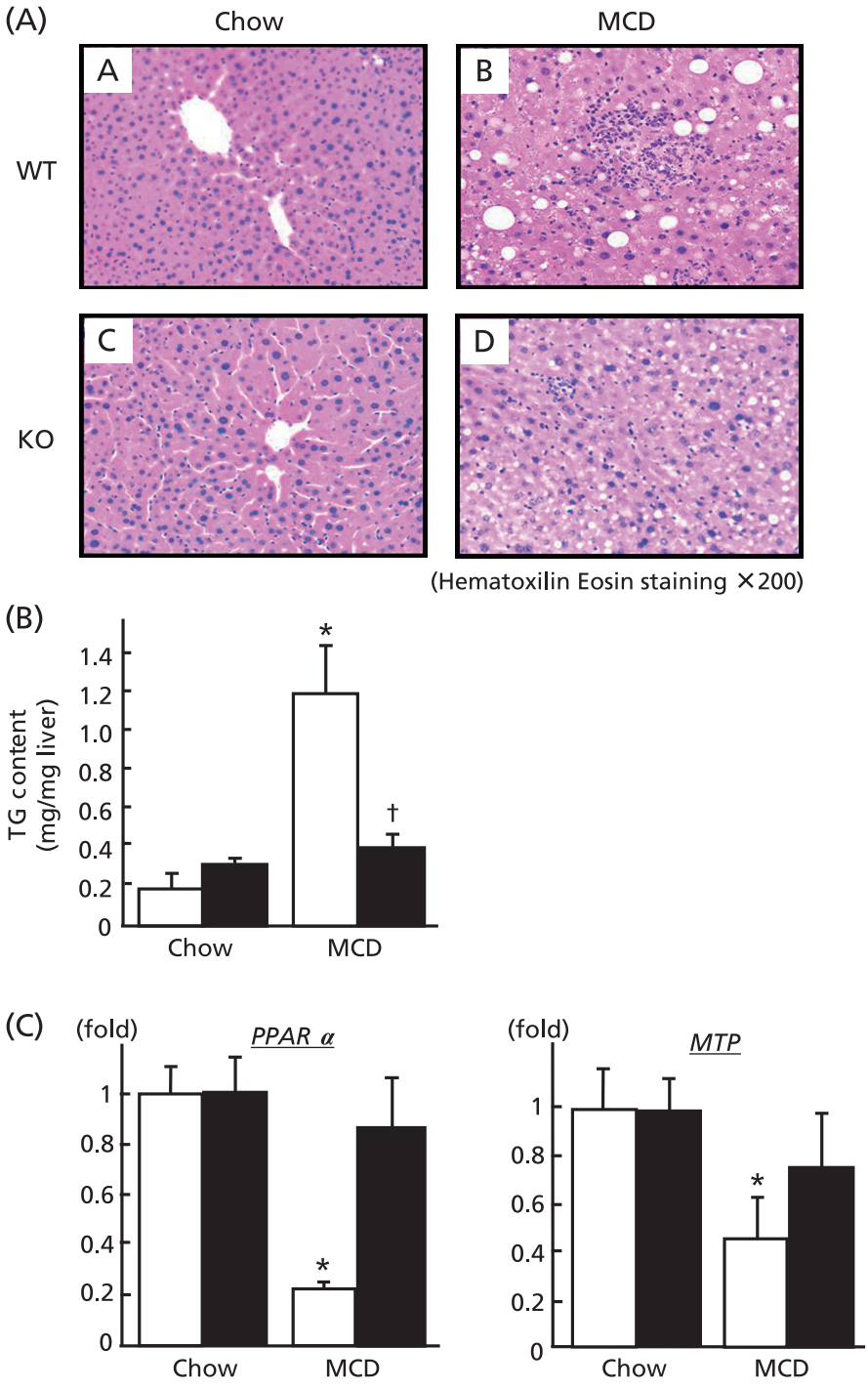

Fig. 2. Influence of Bach1 ablation on hepatic lipid metabolism. (A) Liver sections from wild type (A and B) and Bach1$^{-/-}$ mice (C and D) fed either regular chow (left panels) or MCD diet (right panels) were processed for haematoxylin & eosin (HE) staining. (original magnification 200×). (B) Hepatic triglyceride concentrations in wild type (open bars) and Bach1$^{-/-}$ (closed bars) mice (n = 5/each group) were determined after feeding either regular chow or MCD diet. (C) Hepatic mRNA expressions of PPARα and MTP were quantified (n = 5/each group) by quantitative real-time PCR. *p<0.05, regular chow vs MCD diet. †p<0.05, wild type vs Bach1$^{-/-}$ mice.

triglyceride content increased by 6-fold in wild type mice following MCD diet feeding whereas values remained unchanged in Bach1$^{-/-}$ mice. To further explore the effect of Bach1 ablation, mRNA expressions of key genes involving in hepatic lipid metabolism were investigated. This revealed marked down-regulation of PPARα and MTP in wild type mice whereas there were no significant changes in Bach1$^{-/-}$ mice, suggesting that fatty acid utilization and excretion were preserved in Bach1$^{-/-}$ mice on MCD diet.

Protective effect of Bach1 ablation on MCD diet-induced liver injury. Feeding of MCD diet increased serum ALT levels in wild type mice at 4 and 8 weeks whereas it remained the basal level in Bach1$^{-/-}$ mice, implying protective effect of Bach1 ablation against liver injury (Fig. 3A). Consistent with these findings, hepatic MDA concentration which reflects oxidative damage elicited by lipid peroxidation in the liver remained unchanged in Bach1$^{-/-}$ mice following MCD diet (Fig. 3B). This was in marked

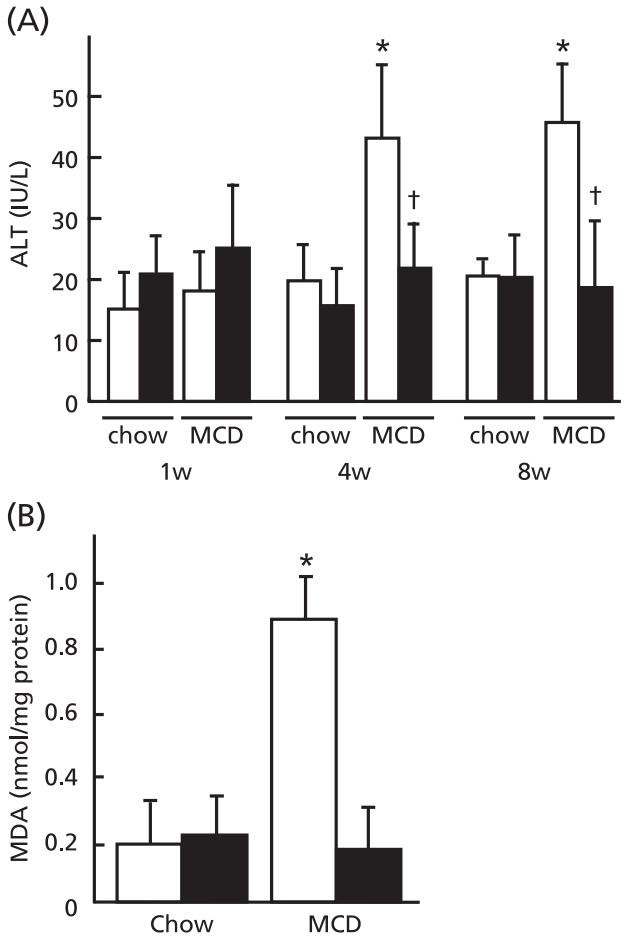

Fig. 3. Protective effect of Bach1 ablation on MCD diet-induced liver injury. (A) Serum ALT levels were determined at indicated time points by 8 w in wild type mice and Bach1$^{-/-}$ mice fed either regular chow or MCD diet (n = 5/each group). (B) Hepatic MDA concentration was assessed with standarlization of protein concentration in wild type (open bar) and Bach1$^{-/-}$ (closed bar) mice (n = 5/each group). *p<0.05, regular chow vs MCD diet. †p<0.05, wild type vs Bach1$^{-/-}$ mice.

contrast to 4-fold increase in MDA level in wild type mice on MCD diet.

Influence of Bach1$^{-/-}$ ablation on hepatic fibrogenesis. As previously reported,[23] rodents fed MCD diet develops steatohepatitis mimicking human NASH. Fig. 4A depicts mild pericellular fibrosis with fat deposition in the liver from wild type mice fed MCD diet (upper panel). In contrast, Bach1$^{-/-}$ mice displayed no significant change in the liver (lower panel). Consistent with these morphological changes, hepatic mRNA expression of α smooth muscle actin (αSMA) which is predominantly expressed in activated stellate cells was up-regulated in the liver of wild type mice on MCD diet while it remained unchanged in Bach1$^{-/-}$ mice.

Discussion

The aim of this study was to explore the influence of Bach1 ablation in the development of NASH. As demonstrated in Fig. 1, Bach1 ablation in mice leads to significant up-regulation of HO-1 mRNA as well as its activity. This was in keeping with previous data that hepatic expressions of HO-1 mRNA and protein were constitutively high in the absence of Bach1.[18] To date, HO-1, β-globin, ferritin, thioredoxin reductase1 and NADP(H) quinine (oxido) reductase have been identified as a target genes of Bach1, but other downstream genes of Bach1 have not been fully understood.[24] HO-1 has been reported to have cytoprotective, anti-inflammatory and anti-oxidative properties. Emerging reports have suggested protective roles of HO-1 in experimental-induced hepatic damage including lipopolysaccharide-induced injury, immune liver fibrosis model, and hepatic ischemia-reperfusion injury.[12,19,25–27] In addition, pathophysiological importance of HO-1 has also been suggested by previous findings that HO-1 is induced under various etiological conditions including human NASH.[14]

The pathogenesis of NASH is widely recognized as two hit theory, in which the first hit is an initial metabolic disturbance causes steatosis and second pathogenic stimulus such as free radical and cytokines triggers necro/inflammation leading to the progression of steatohepatitis. The current study was undertaken on the basis of our initial hypothesis that HO-1 induction by Bach1 ablation may ameliorate oxidative stress and inflammation, the second hit in NASH pathogenesis and may repress the transition from bland steatosis to steatohepatitis. Surprisingly, in addition to reduction in hepatic damage, Bach1 ablation significantly diminished hepatic steatosis following MCD feeding. This was explained by the finding in this study that hepatic expression of PPARα and MTP, that are involved in hepatic fatty acid oxidation and hepatic secretion of triglyceride-rich VLDL, remained unchanged in MCD diet-fed Bach1$^{-/-}$ mice in comparison to significant reduction in wild type mice. These changes were inversely paralleled with hepatic MDA concentrations, suggesting the possibility that relief from oxidative damage in Bach1$^{-/-}$ mice conserved hepatic lipid metabolism. Along a similar line, the previous study of ob/ob mice fed MCD diet has revealed that YHK, a naturally derived herbal medicine that is reported to reduce reactive oxygen species declined hepatic steatosis via induction of hepatic mRNAs of PPARα and MTP.[28,29] This suggests a potential mechanism of the previous findings that antioxidant agents have beneficial effects not only on hepatic necro/inlflammation but also on hepatic steatosis.[4,28] Since mice assigned to high fat diet or dietary choline restriction exhibited mitochondrial dysfunction which negatively affects the bioenergenetics of liver, antioxidative status observed in Bach$^{-/-}$ mice might preserve the integrity of the mitochondria from oxidative damage and help to maintain cellular functions including hepatic lipid metabolism.[30,31] However, the current study lacks the direct evidence that hepatoprotective property against NASH in Bach1$^{-/-}$ mice was due to reduction in oxidative stress attributed to HO-1 induction, and further investigations are needed.

As shown in Fig. 4, MCD diet caused modest pericellular fibrosis with significant induction of αSMA mRNA in wild type mice whereas Bach1$^{-/-}$ mice exhibited almost normal in liver histology as well as αSMA expression. Reduction in hepatic fibrosis is likely to be elicited by relief of hepatic damage and inflammation in Bach1$^{-/-}$ mice. However, previous report has demonstrated that HO-1 is expressed in human hepatic myofibroblast and induction of HO-1 leads to inhibition of collagen synthesis and to reduction in proliferation of myofibroblast.[32] This suggests direct role for HO-1 induction against the development of hepatic fibrosis and might be a possible explanation for our finding.

In the current study, MCD dietary model was utilized as a NASH animal model. Although this model is widely used on the basis that morphological changes of the liver resembles human NASH with induction of oxidative stress, this does not entirely reflect the etiological background of human NASH such as obesity and insulin resistance. Alternative dietary models for NASH include high fat diet, which develops obesity and insulin resistance but not remarkable steatohepatitis as seen in MCD diet model.[33] Interestingly, recent study has reported that feeding rats with choline-deficient fat-rich diet for 8 w lead to marked steatohepatitis.[34]

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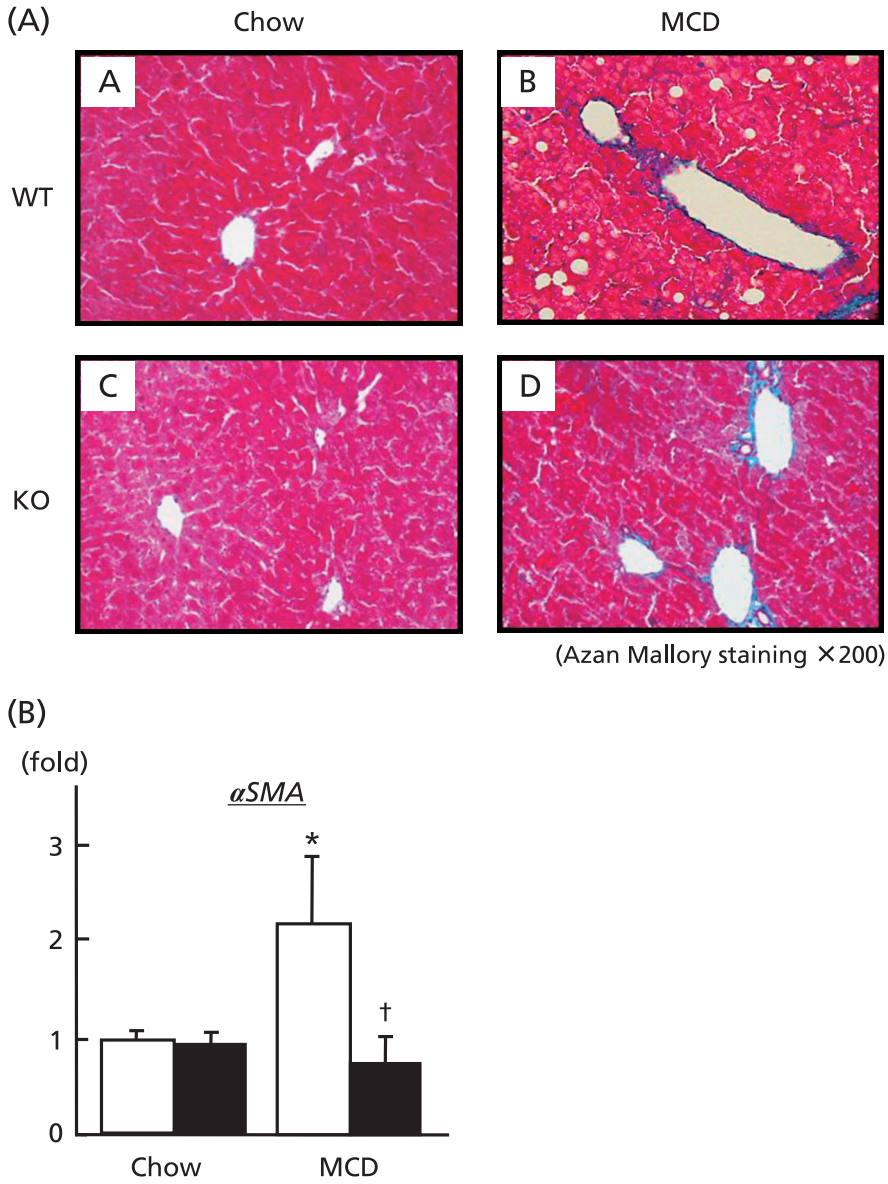

Fig. 4. Influence of Bach1$^{-/-}$ ablation on hepatic fibrogenesis. (A) Liver sections from wild type (A and B) and Bach1$^{-/-}$ mice (C and D) fed either regular chow (left panels) or MCD diet (right panels) were processed for Azan-Mallory staining. (original magnification 200×). (B) Following 4 w of either regular chow or MCD diet, hepatic mRNA expressions of αSMA were quantified (n = 5/each group) by quantitative real-time PCR. *p<0.05, regular chow vs MCD diet. †p<0.05, wild type (open bar) vs Bach1$^{-/-}$ mice (closed bar).

In conclusion, Bach1$^{-/-}$ mice are resistant to the development of hepatic steatohepatitis with concomitant induction of HO-1 in MCD diet-induced NASH model. Reduced steatosis in Bach1$^{-/-}$ mice following MCD diet might be attributable to preserved expressions of PPARα and MTP that play an important role in hepatic fatty acid oxidation and triglyceride secretion respectively. Hepatic levels of MDA, a biomarker of oxidative stress, as well as serum ALT, an indicator of liver injury were significantly decreased Bach1$^{-/-}$ mice compared to wild type mice, suggesting anti-oxidative effect of HO-1 induction in the absence of Bach1. Since oxidative stress, the second hit in NASH pathogenesis causes impairment of cellular bioenergetics leading to further disruption of hepatic lipid metabolism, abatement of oxidative stress by Bach1 inhibition might be one of therapeutic strategies for the treatment of NASH/NAFLD.

Acknowlegment

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Abbreviations

HO	heme oxygenase
NASH	nonalcoholic steatohepatitis
MCD diet	methionine-choline deficient diet
PPARα	peroxisome proliferator-activated receptor α
MTP	microsomal triglyceride transfer protein
MDA	malondialdehyde
ALT	alanine aminotransferase
NAFLD	nonalcoholic fatty liver disease
CO	carbon monoxide

CoPP	cobalt protoporrphyrin
Nrf2	NF-E2 related factor
MAREs	Maf recognition elements
HE	hematoxylin and eosin
ZnPP	zinc protoporphyrin
αSMA	alpha smooth muscle actin

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