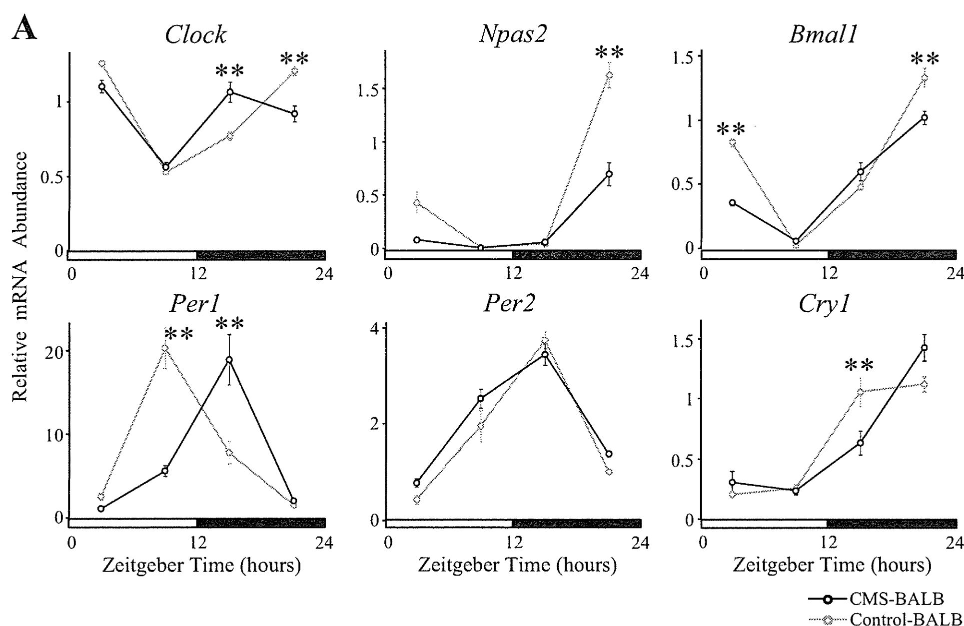


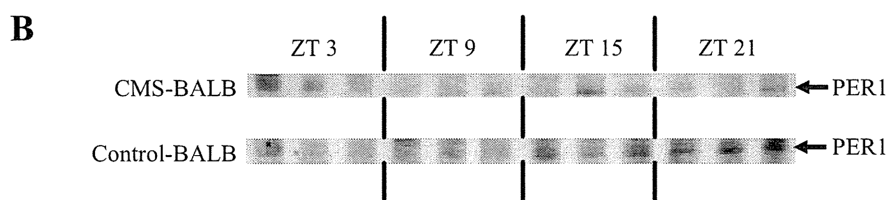


Fig. 3. Transcripts of circadian clock genes in the livers of BALB/c mice. *A*: expressions of mRNAs encoding *Clock*, *NPAS2*, *Bmal1*, *Per1*, *Per2*, and *Cry1* in the livers of Control-BALB and CMS-BALB mice; n = 6/group for each observation point. Results are expressed as means ± SE. Two-way ANOVA was used to determine variance with respect to time and groups, followed by Bonferroni's post hoc test (**$P < 0.01$). *B*: immunoblotting of the liver extracts of Control-BALB and CMS-BALB mice with anti-PER1 antibody.

sterone levels (38). Therefore, we examined the effects of CMS on hepatic core clock gene expressions in C57BL/6 mice.

C57BL/6 mice were divided into two groups in a manner similar to that used for BALB/c mice; one group was housed under ordinary conditions (Control-C57), and the other was subjected to the same CMS (CMS-C57). Consistent with a previous report (38), Control-C57 and CMS-C57 mice showed no significant differences in serum corticosterone levels or rhythms (Fig. 5*A*), indicating that CMS does not affect the HPA axis in these mice. Body weight, blood glucose, serum insulin, and leptin levels remained unchanged (Fig. 5*B*). No significant differences in food intake amounts were observed between CMS-C57 and Control-C57 mice during either the light or the dark phase of the day after the last day of the CMS

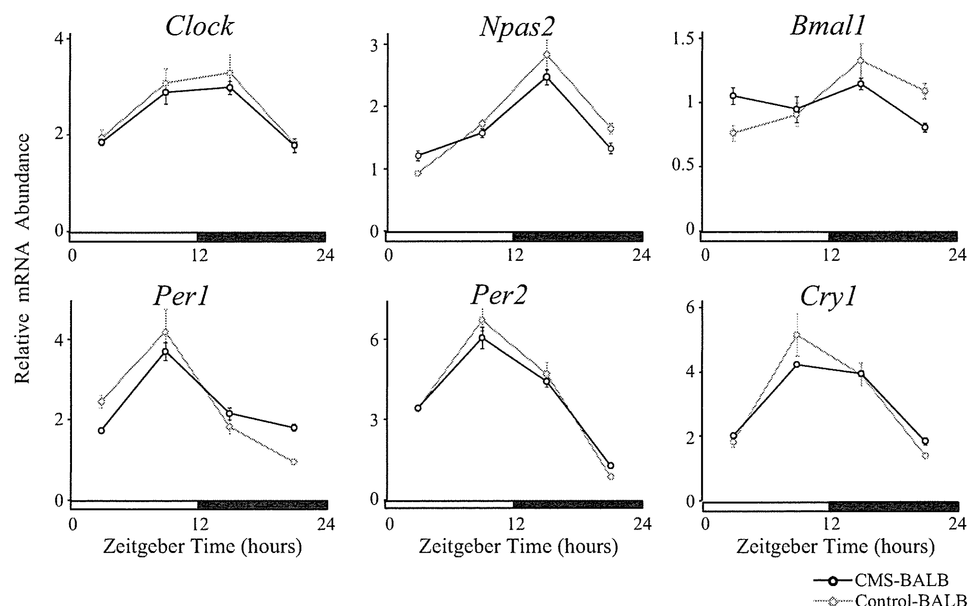


Fig. 4. Transcripts of circadian clock genes in the hypothalamic suprachiasmatic nucleus (SCN) of BALB/c mice. Expressions of mRNAs encoding *Clock*, *NPAS2*, *Bmal1*, *Per1*, *Per2*, and *Cry1* in the SCN of Control-BALB and CMS-BALB mice; n = 6/group for each observation point. Results are expressed as means ± SE. Two-way ANOVA was used to determine variance with respect to time and groups, followed by Bonferroni's post hoc test.

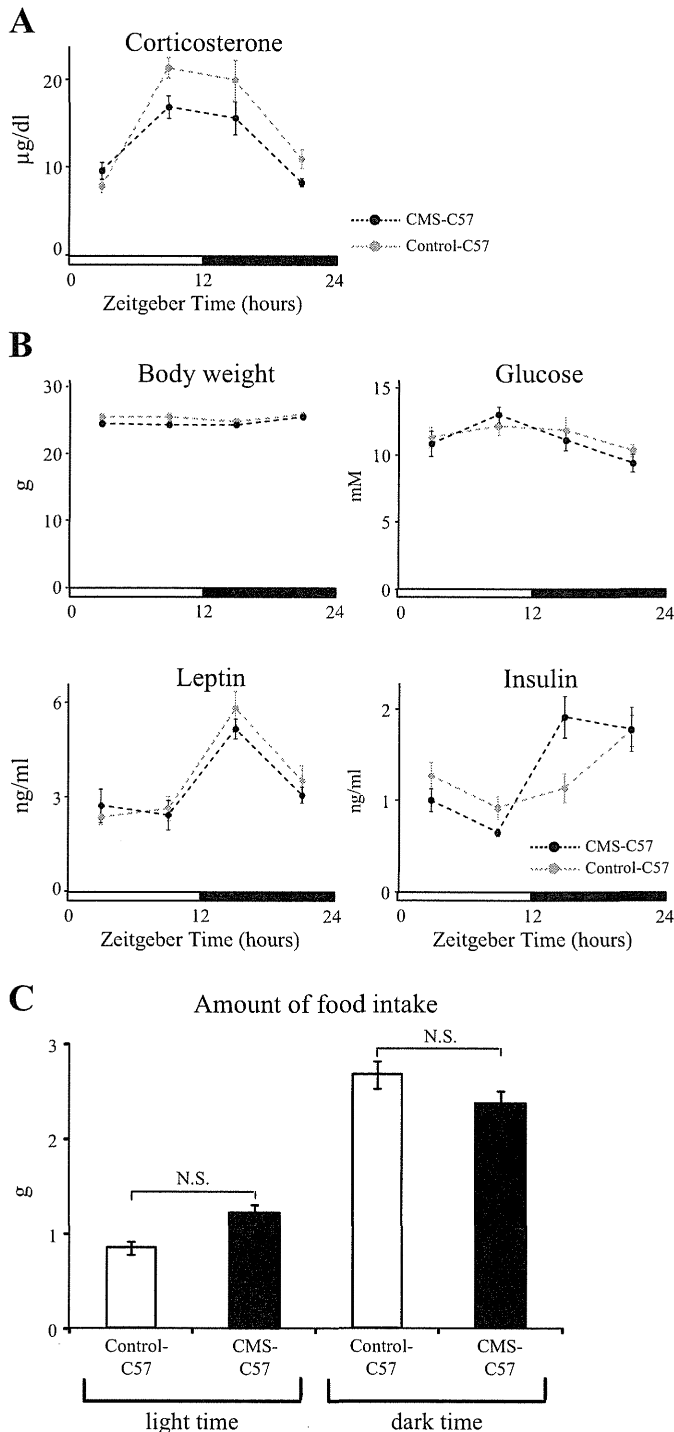


Fig. 5. Metabolic parameters of C57BL/6 mice. Mice were assigned to either the Control-C57 or the CMS-C57 group. Diurnal variations in plasma corticosterone (*A*), body weight, blood glucose, plasma insulin, and leptin (*B*) are shown; n = 6/group for each observation point. Results are expressed as means ± SE. Two-way ANOVA was used to determine variance with respect to time and groups, followed by Bonferroni's post hoc test. *C*: food intake was measured 1 day after the last day of the CMS procedure, with division into light and dark phases. Results are expressed as means ± SE. One-way ANOVA was used to determine statistical significance.

procedure (Fig. 5*C*). In contrast to BALB/c mice, even under CMS, the rhythms and expressions of *Clock*, *Npas2*, *Bmal1*, *Per1*, *Per2*, and *Cry1* were not significantly altered in the liver (Fig. 6). These results suggest that HPA axis activation itself, rather than each of the zeitgebers comprising the CMS procedure, plays a major role in altered circadian expressions of hepatic clock genes.

HPA axis activation alters circadian mRNA expression profiles of metabolism-related genes in the livers of BALB/c mice. Previous reports have demonstrated the liver clock to function in vivo and participate in glucose and lipid homeostasis (29, 30). Therefore, we next examined the expression rhythms of genes involved in glucose and lipid metabolism in the livers of Control-BALB, CMS-BALB, Control-C57, and CMS-C57 mice. We first analyzed the circadian expression profiles of *Pparα*, *Pparγ-1*, and *Pgc-1α*, which are involved in lipid metabolism and known to be rhythmically expressed in the liver (14). CMS significantly altered the hepatic circadian expressions of all these molecules in BALB/c (2-way ANOVA; F = 5.7232, $P = 3.2 \times 10^{-3}$ for *Pparα*; F = 24.8525, $P < 1.0 \times 10^{-4}$ for *Pparγ-1*; F = 17.0444, $P < 1.0 \times 10^{-4}$ for *Pgc-1α*) but not in C57BL/6 mice (Fig. 7*A*). In addition, the expression rhythm of *Pepck* (2-way ANOVA; F = 17.2246, $P < 1.0 \times 10^{-4}$), which is the rate-limiting enzyme in gluconeogenesis, of which expression is regulated by PGC-1α (20), was markedly altered in CMS-BALB mice (Fig. 7*B*). These results suggest an important role of HPA axis activation in the expressions of these glucose- and lipid metabolism-related genes under chronic stress conditions. In contrast, CMS altered the circadian expressions of *glucose-6-phosphatase* (*G6Pase*) in both BALB/c (2-way ANOVA; F = 7.3724, $P = 6.0 \times 10^{-4}$) and C57BL/6 mice (2-way ANOVA; F = 8.6569, $P = 3.0 \times 10^{-4}$) (Fig. 7*B*). Interestingly, this CMS procedure did not change hepatic expression profiles of *Srebp1c* and *Fas*, both of which mediate lipogenesis, in BALB/c mice, whereas their expressions were altered in C57BL/6 mice (2-way ANOVA; F = 4.9484, $P = 6.8 \times 10^{-3}$ for *Srebp1c*; F = 17.5981, $P < 1.0 \times 10^{-4}$ for *Fas*) (Fig. 7*C*). These results indicate that this CMS procedure actually affects gene expressions in the livers of C57BL/6 mice but that circadian expressions of lipogenesis-related genes in the liver are disturbed via a pathway(s) other than the HPA axis. Collectively, these findings suggest that chronic stress affects profiles of the rhythms of glucose- and lipid metabolism-related gene expressions in the liver via distinct mechanisms.

DISCUSSION

In this study, to examine whether chronic stress modifies circadian rhythms of hepatic clock genes, we chose a CMS model that has been widely used for producing a state of chronic stress (34, 38, 39, 53). This CMS procedure consists of stressors, including water deprivation, food deprivation, and 12-h overnight illumination, all of which can affect circadian expression rhythms of clock genes in peripheral tissues (11, 32). In a state of chronic stress, hepatic clock alterations were observed in BALB/c mice but not in C57BL/6 mice. These results indicate that each stressor cue by itself does not affect the rhythms of hepatic clock expressions. Rather, the modulated physiological responses to these stress cues affect hepatic oscillation. Such responses are thought to be adaptive pro-

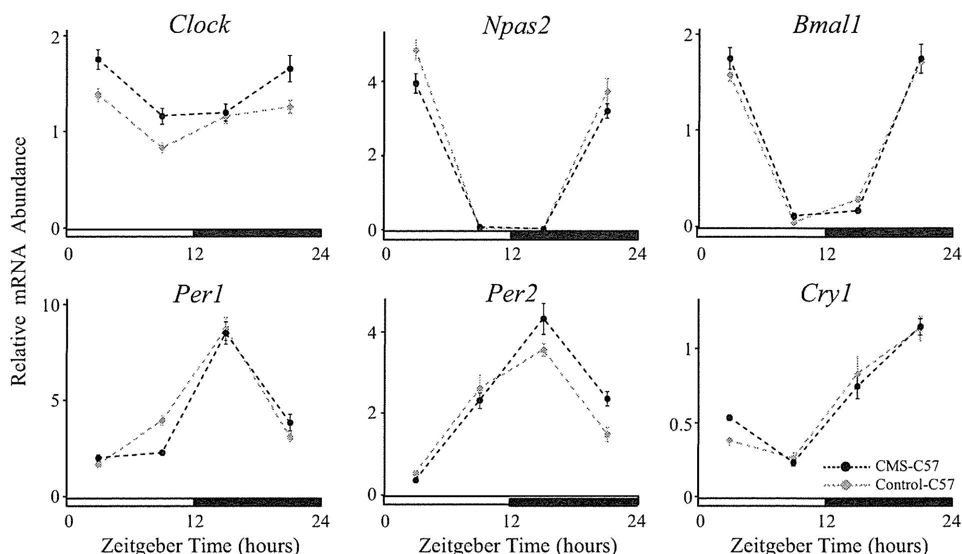


Fig. 6. Transcripts of circadian clock genes in the livers of C57BL/6 mice. Expressions of mRNAs encoding *Clock*, *NPAS2*, *Bmal1*, *Per1*, *Per2*, and *Cry1* in the livers of Control-C57 and CMS-C57 mice; $n = 6$/group for each observation point. Results are expressed as means ± SE. Two-way ANOVA was used to determine variance with respect to time and groups, followed by Bonferroni's post hoc test.

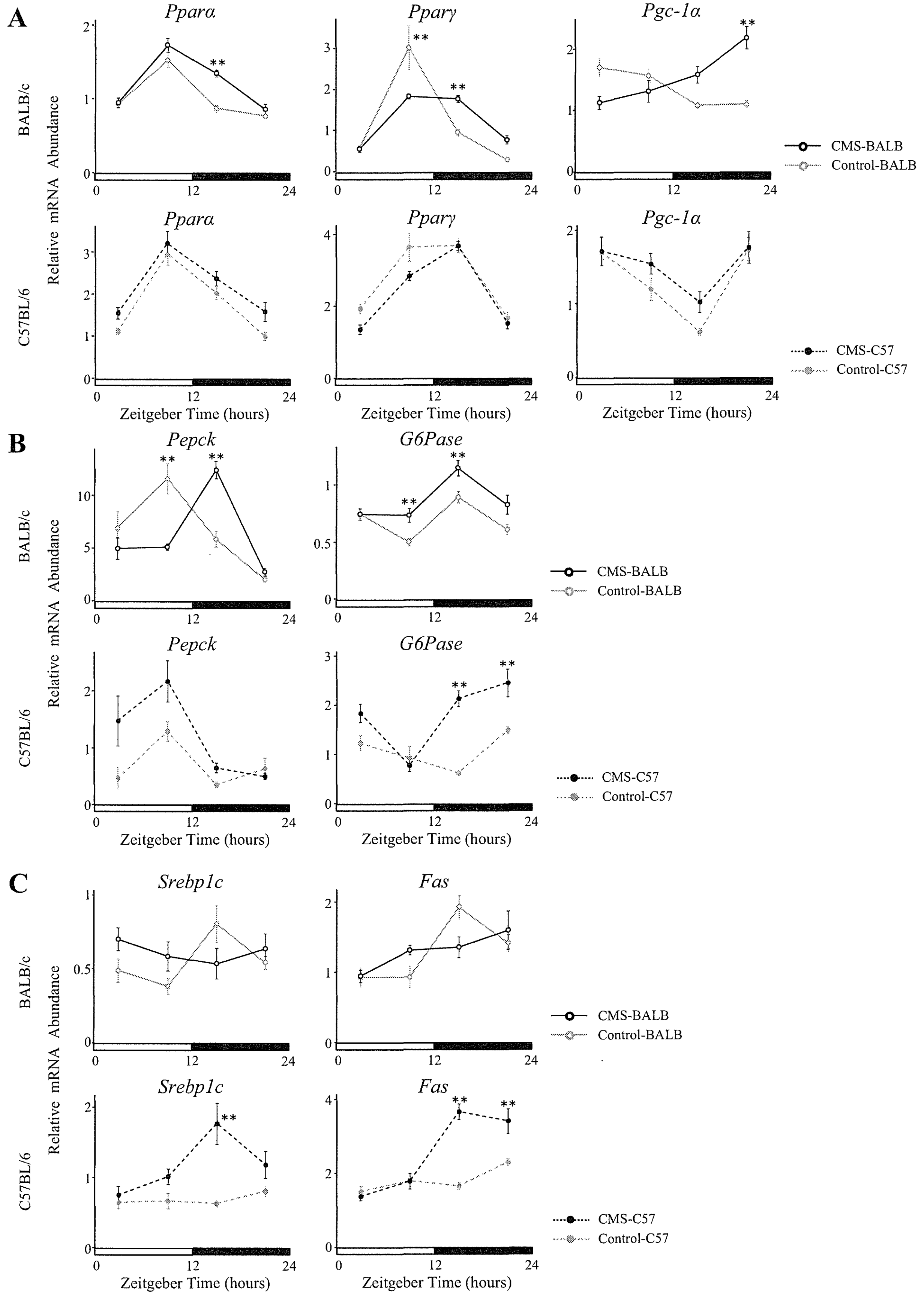
cesses that maintain homeostasis via the HPA axis and the autonomic nervous system (35). A previous study demonstrated that this CMS procedure activates the HPA axis in BALB/c mice but not in C57BL/6 mice (38). These findings are consistent with our observations that this CMS procedure raises serum corticosterone levels in BALB/c mice but not in C57BL/6 mice. On the other hand, circadian expressions of *Srebp1c* and *Fas* in the liver were remarkably changed in C57BL/6 mice, indicating that these stress cues function as zeitgebers in peripheral organs, including the liver, via a pathway other than the HPA axis. We cannot rule out the possibility that the slight, not statistically significant, suppression of corticosterone levels observed in CMS-C57 mice affects circadian expressions of these lipogenesis-related genes. However, corticosterone elevations in BALB/c mice did not significantly affect the expressions of these genes. In addition, CMS altered neither the rhythms nor the expressions of *Clock*, *Npas2*, *Bmal1*, *Per1*, *Per2*, and *Cry1* in the livers of C57BL/6 mice. In particular, given that stressed BALB/c mice displayed serum corticosterone elevations and altered circadian clock gene expressions before exhibiting any remarkable metabolic alterations in body weight and blood glucose, it is likely that overactivation of the HPA axis itself, i.e., without any metabolic disorder phenotype, is sufficient to alter the circadian clock system in response to CMS. Moreover, dysregulations of metabolism-related genes, such as *Pparα*, *Pparγ-1*, *Pgc-1α*, and *Pepck*, in the liver were observed in stressed BALB/c mice. These results raise the possibility that alterations in the circadian clock system are among the triggers of metabolic syndrome onset associated with chronic stress.

This CMS procedure includes 12-h overnight illumination. The SCN master clock receives direct photic input via the retinohypothalamic tract, and the presence of light is known to be the key environmental timing cue that entrains the core clock genes in the SCN (48). However, circadian expressions of these genes in the SCN were not affected by the CMS procedure in BALB/c mice. This finding further supports the importance of the HPA axis in the regulation of hepatic clock oscillation.

Recent studies indicate that the SCN affects the circadian pattern of corticosterone secretion (25). In the present study, however, CMS did not alter rhythms in the SCN. One of the plausible mechanisms whereby CMS alters the daily pattern of corticosterone secretion without altering SCN rhythms is limbic regulation of the HPA axis. Limbic regulation of the HPA axis is achieved through relaying neuronal inputs from limbic regions of the brain, such as the hippocampus and amygdala, to the paraventricular nucleus (PVN) of the hypothalamus (22). PVN releases corticotropin-releasing hormone (CRH), and CRH in turn stimulates pituitary release of adrenocorticotropic hormone (ACTH). ACTH then acts upon cells in the adrenal cortex to augment the synthesis and secretion of glucocorticoids. Therefore, CMS can alter the daily pattern of corticosterone secretion via the limbic system without involving the SCN.

It is well known that glucocorticoids, which act as end effectors of the HPA axis, reset the circadian rhythms of peripheral organs, including the liver, by shifting the phase of circadian gene expressions (7, 35). In vitro studies have demonstrated that glucocorticoids stimulate the transcriptional rhythmicities of *Per1* (5), *NPAS2* (45), *Cry1* (5), *Bmal1*, and *Clock* (36), all of which were found to show altered oscillations in the present study. Glucocorticoid receptors bind to glucocorticoids and occupy the glucocorticoid-responsive element (GRE) in the genome, thereby regulating target gene transcription (45). The GRE is located in the promoter of *Per1* and is reportedly involved in *Per1* expression (54), which allows

Fig. 7. Transcripts involved in lipid and glucose metabolism in the livers of BALB/c and C57BL/6 mice. Expressions of mRNAs encoding *peroxisome proliferator activated receptor* (*Ppar*) α, *Pparγ*, and *Pparγ-coactivator-1α* (*Pgc-1α*) (*A*), *phosphoenolepyruvate carboxykinase* (*Pepck*) and *glucose-6-phosphatase* (*G6Pase*) (*B*), and *sterol regulatory element-binding protein 1c* (*Srebp1c*) and *fatty acid synthase* (*Fas*) (*C*) in the livers of Control-BALB, CMS-BALB, Control-C57, and CMS-C57 mice; $n = 6$/group for each observation point. Results are expressed as means ± SE. Two-way ANOVA was used to determine variance with respect to time and groups, followed by Bonferroni's post hoc test ($^{**}P < 0.01$).


A
B
C
Pparα
Pparγ
Pgc-1α
Pepck
G6Pase
Srebp1c
Fas
BALB/c
C57BL/6
Relative mRNA Abundance
Zeitgeber Time (hours)
CMS-BALB
Control-BALB
CMS-C57
Control-C57
**

glucocorticoids to directly interact with PER1. Therefore, upregulation of glucocorticoids induced by overactivation of the HPA axis might directly affect the circadian expressions of core clock genes in the liver.

Persistent stimulation of the HPA axis by various stressors causes metabolic disorders, such as insulin resistance/overt diabetes mellitus, hypertension, and dyslipidemia (7, 35). Interestingly, these metabolic disturbances are also induced by dysregulation of the clock system (6, 14). However, whether modulation of the HPA axis leads to metabolic problems through disruption of the clock system, or these systems influence the same metabolic pathway independently, has not been determined. Several molecules, such as PPARα, PPARγ and PGC-1α, play crucial roles in intermediary metabolism and respond to both the clock system and glucocorticoids (6, 35). In the liver, BMAL1 (8) and CLOCK (37) have both been demonstrated to be upstream regulators of *Pparα* gene expression. CRY1 regulates the activity of cAMP response element-binding (CREB) protein (56), and CREB in turn inhibits hepatic PPARγ (18) or enhances hepatic PGC-1α expression (19). Consistent with these reports, we observed perturbed rhythms of the expressions of core clock genes, including *Bmal1*, *Clock*, and *Cry1*, and metabolism-related genes, including *Pparα*, *Pparγ*, and *Pgc-1α*, in the livers of CMS-BALB mice. Although PER2 has been shown in adipocytes to directly repress PPARγ (15), hepatic expression rhythms of *Per2* were not altered in the present study. Therefore, altered expressions of *Per2* are not required for CMS-induced perturbation of metabolism-related gene expressions in the liver. Furthermore, rhythms of *Pepck* expressions were also affected in the livers of CMS-BALB mice. PGC-1α mediates the induction of PEPCK by glucocorticoids (19). Thus, in a chronically stressed state, the overactivated HPA axis acts together with the clock system, which may lead to the onset of metabolic disturbance in the liver.

Recent studies have suggested that rhythmic abnormalities affect energy homeostasis as well as glucose and lipid metabolism (40, 42, 50). Genetically obese strains such as KK-Ay (3) and *db/db* mice (28), and mice with high-fat diet-induced obesity (27), exhibit attenuated circadian expressions of several clock genes in the liver. Intriguingly, in all of these murine models, serum corticosterone levels were shown to be altered (1, 27). In addition, circadian expressions of hepatic clock genes are impaired in young *ob/ob* mice even before the onset of metabolic abnormalities, and this impairment in rhythmic expressions of hepatic clock genes was restored by leptin treatment (2). Serum corticosterone levels are increased in *ob/ob* mice (1, 52), and leptin treatment depresses corticosterone levels via suppression of the HPA axis (17). Taking these previous reports and our present results together, HPA axis activation may contribute to alterations in circadian clock gene expressions in the liver, not only under chronic stress conditions, but also in states of excess nutrition. This speculation is supported by several reports in which high fat diets exaggerated the responsiveness of the HPA axis to chronic stress (31, 49).

In summary, to our knowledge, this is the first report showing that chronic stress perturbs the circadian expressions of clock genes in the liver. Overactivaton of the HPA axis is likely to play an important role in the underlying mechanism. The dysregulation of circadian expressions of genes regulating metabolism might trigger metabolic disturbances in states of chronic stress.

ACKNOWLEDGMENTS

We thank M. Hoshi, T. Takasugi, J. Fushimi, and R. Tomono for technical assistance.

GRANTS

This work was supported by Grants-in-aid for Scientific Research (C, 23591293 to T. Yamada and B2, 15390282 to H. Katagiri) from the Japan Society for the Promotion of Science. This work was also supported by Global-COE (H. Katagiri and Y. Oka) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

DISCLOSURES

The authors have no conflicts of interest, financial or otherwise, to declare.

AUTHOR CONTRIBUTIONS

Author contributions: K.T. T.Y. and H.K. conception and design of research; K.T. T.Y. and H.K performed experiments; K.T., T.Y., and H.K. analyzed data; K.T., T.Y., S.T., K.K., Y.S., Y.M., Y.I., J.I., K.U., Y.H., S.S., Y.O., and H.K. interpreted results of experiments; K.T. prepared figures; K.T., T.Y., and H.K. drafted manuscript; K.T., T.Y., and H.K. edited and revised manuscript; K.T., T.Y., S.T., K.K., Y.S., Y.M., Y.I., J.I., K.U., Y.H., S.S., Y.O., and H.K. approved final version of manuscript.

REFERENCES

1. **Alberts P, Ronquist-Nii Y, Larsson C, Klingstrom G, Engblom L, Edling N, Lidell V, Berg I, Edlund PO, Ashkzari M, Sahaf N, Norling S, Berggren V, Bergdahl K, Forsgren M, Abrahmsen L.** Effect of high-fat diet on KKAy and ob/ob mouse liver and adipose tissue corticosterone and 11-dehydrocorticosterone concentrations. *Horm Metab Res* 37: 402–407, 2005.
2. **Ando H, Kumazaki M, Motosugi Y, Ushijima K, Maekawa T, Ishikawa E, Fujimura A.** Impairment of peripheral circadian clocks precedes metabolic abnormalities in ob/ob mice. *Endocrinology* 152: 1347–1354, 2011.
3. **Ando H, Oshima Y, Yanagihara H, Hayashi Y, Takamura T, Kaneko S, Fujimura A.** Profile of rhythmic gene expression in the livers of obese diabetic KK-A(y) mice. *Biochem Biophys Res Commun* 346: 1297–1302, 2006.
4. **Ando H, Yanagihara H, Hayashi Y, Obi Y, Tsuruoka S, Takamura T, Kaneko S, Fujimura A.** Rhythmic messenger ribonucleic acid expression of clock genes and adipocytokines in mouse visceral adipose tissue. *Endocrinology* 146: 5631–5636, 2005.
5. **Balsalobre A, Brown SA, Marcacci L, Tronche F, Kellendonk C, Reichardt HM, Schutz G, Schibler U.** Resetting of circadian time in peripheral tissues by glucocorticoid signaling. *Science* 289: 2344–2347, 2000.
6. **Bass J, Takahashi JS.** Circadian integration of metabolism and energetics. *Science* 330: 1349–1354, 2010.
7. **Cagampang FR, Poore KR, Hanson MA.** Developmental origins of the metabolic syndrome: body clocks and stress responses. *Brain Behav Immun* 25: 214–220, 2011.
8. **Canaple L, Rambaud J, Dkhissi-Benyahya O, Rayet B, Tan NS, Michalik L, Delaunay F, Wahli W, Laudet V.** Reciprocal regulation of brain and muscle Arnt-like protein 1 and peroxisome proliferator-activated receptor alpha defines a novel positive feedback loop in the rodent liver circadian clock. *Mol Endocrinol* 20: 1715–1727, 2006.
9. **Chrousos GP.** Stress and disorders of the stress system. *Nat Rev Endocrinol* 5: 374–381, 2009.
10. **Chung S, Son GH, Kim K.** Circadian rhythm of adrenal glucocorticoid: its regulation and clinical implications. *Biochim Biophys Acta* 1812: 581–591, 2011.
11. **Dibner C, Schibler U, Albrecht U.** The mammalian circadian timing system: organization and coordination of central and peripheral clocks. *Annu Rev Physiol* 72: 517–549, 2010.
12. **Dunlap JC.** Running a clock requires quality time together. *Science* 311: 184–186, 2006.
13. **Field MD, Maywood ES, O'Brien JA, Weaver DR, Reppert SM, Hastings MH.** Analysis of clock proteins in mouse SCN demonstrates

phylogenetic divergence of the circadian clockwork and resetting mechanisms. *Neuron* 25: 437–447, 2000.

14. **Froy O.** Metabolism and circadian rhythms–implications for obesity. *Endocr Rev* 31: 1–24, 2010.
15. **Grimaldi B, Bellet MM, Katada S, Astarita G, Hirayama J, Amin RH, Granneman JG, Piomelli D, Leff T, Sassone-Corsi P.** PER2 controls lipid metabolism by direct regulation of PPARgamma. *Cell Metab* 12: 509–520, 2000.
16. **Hastings MH, Field MD, Maywood ES, Weaver DR, Reppert SM.** Differential regulation of mPER1 and mTIM proteins in the mouse suprachiasmatic nuclei: new insights into a core clock mechanism. *J Neurosci* 19: RC11, 1999.
17. **Heiman ML, Ahima RS, Craft LS, Schoner B, Stephens TW, Flier JS.** Leptin inhibition of the hypothalamic-pituitary-adrenal axis in response to stress. *Endocrinology* 138: 3859–3863, 1997.
18. **Herzig S, Hedrick S, Morantte I, Koo SH, Galimi F, Montminy M.** CREB controls hepatic lipid metabolism through nuclear hormone receptor PPAR-gamma. *Nature* 426: 190–193, 2003.
19. **Herzig S, Long F, Jhala US, Hedrick S, Quinn R, Bauer A, Rudolph D, Schutz G, Yoon C, Puigserver P, Spiegelman B, Montminy M.** CREB regulates hepatic gluconeogenesis through the coactivator PGC-1. *Nature* 413: 179–183, 2001.
20. **Herzog B, Hall RK, Wang XL, Waltner-Law M, Granner DK.** Peroxisome proliferator-activated receptor gamma coactivator-1alpha, as a transcription amplifier, is not essential for basal and hormone-induced phosphoenolpyruvate carboxykinase gene expression. *Mol Endocrinol* 18: 807–819, 2004.
21. **Honma S, Kawamoto T, Takagi Y, Fujimoto K, Sato F, Noshiro M, Kato Y, Honma K.** Dec1 and Dec2 are regulators of the mammalian molecular clock. *Nature* 419: 841–844, 2002.
22. **Howell MP, Muglia LJ.** Effects of genetically altered brain glucocorticoid receptor action on behavior and adrenal axis regulation in mice. *Front Neuroendocrinol* 27: 275–284, 2006.
23. **Huang W, Ramsey KM, Marcheva B, Bass J.** Circadian rhythms, sleep, and metabolism. *J Clin Invest* 121: 2133–2141, 2011.
24. **Ibarguen-Vargas Y, Surget A, Touma C, Palme R, Belzung C.** Multifaceted strain-specific effects in a mouse model of depression and of antidepressant reversal. *Psychoneuroendocrinology* 33: 1357–1368, 2008.
25. **Kalsbeek A, van der Spek R, Lei J, Endert E, Buijs RM, Fliers E.** Circadian rhythms in the hypothalamo-pituitary-adrenal (HPA) axis. *Mol Cell Endocrinol* 349: 20–29, 2000.
26. **Kaneko K, Yamada T, Tsukita S, Takahashi K, Ishigaki Y, Oka Y, Katagiri H.** Obesity alters circadian expressions of molecular clock genes in the brainstem. *Brain Res* 1263: 58–68, 2009.
27. **Kohsaka A, Laposky AD, Ramsey KM, Estrada C, Joshu C, Kobayashi Y, Turek FW, Bass J.** High-fat diet disrupts behavioral and molecular circadian rhythms in mice. *Cell Metab* 6: 414–421, 2007.
28. **Kudo T, Akiyama M, Kuriyama K, Sudo M, Moriya T, Shibata S.** Night-time restricted feeding normalises clock genes and Pai-1 gene expression in the db/db mouse liver. *Diabetologia* 47: 1425–1436, 2004.
29. **Lamia KA, Storch KF, Weitz CJ.** Physiological significance of a peripheral tissue circadian clock. *Proc Natl Acad Sci USA* 105: 15172–15177, 2008.
30. **Le Martelot G, Claudel T, Gatfield D, Schaad O, Kornmann B, Sasso GL, Moschetta A, Schibler U.** REV-ERBalpha participates in circadian SREBP signaling and bile acid homeostasis. *PLoS Biol* 7: e1000181, 2009.
31. **Legendre A, Harris RB.** Exaggerated response to mild stress in rats fed high-fat diet. *Am J Physiol Regul Integr Comp Physiol* 291: R1288–R1294, 2006.
32. **Liu AC, Lewis WG, Kay SA.** Mammalian circadian signaling networks and therapeutic targets. *Nat Chem Biol* 3: 630–639, 2007.
33. **Marcheva B, Ramsey KM, Buhr ED, Kobayashi Y, Su H, Ko CH, Ivanova G, Omura C, Mo S, Vitaterna MH, Lopez JP, Philipson LH, Bradfield CA, Crosby SD, JeBailey L, Wang X, Takahashi JS, Bass J.** Disruption of the clock components CLOCK and BMAL1 leads to hypoinsulinaemia and diabetes. *Nature* 466: 627–631, 2010.
34. **Monleon S, D'Aquila P, Parra A, Simon VM, Brain PF, Willner P.** Attenuation of sucrose consumption in mice by chronic mild stress and its restoration by imipramine. *Psychopharmacology (Berl)* 117: 453–457, 1995.
35. **Nader N, Chrousos GP, Kino T.** Interactions of the circadian CLOCK system and the HPA axis. *Trends Endocrinol Metab* 21: 277–286, 2010.
36. **Nebzydoski SJ, Pozzo S, Nemec L, Rankin MK, Gressley TF.** The effect of dexamethasone on clock gene mRNA levels in bovine neutrophils and lymphocytes. *Vet Immunol Immunopathol* 138: 183–192, 2010.
37. **Oishi K, Kasamatsu M, Ishida N.** Gene- and tissue-specific alterations of circadian clock gene expression in streptozotocin-induced diabetic mice under restricted feeding. *Biochem Biophys Res Commun* 317: 330–334, 2004.
38. **Palumbo ML, Canzobre MC, Pascuan CG, Rios H, Wald M, Genaro AM.** Stress induced cognitive deficit is differentially modulated in BALB/c and C57Bl/6 mice: correlation with Th1/Th2 balance after stress exposure. *J Neuroimmunol* 218: 12–20, 2010.
39. **Palumbo ML, Fosser NS, Rios H, Zorrilla Zubilete MA, Guelman LR, Cremaschi GA, Genaro AM.** Loss of hippocampal neuronal nitric oxide synthase contributes to the stress-related deficit in learning and memory. *J Neurochem* 102: 261–274, 2007.
40. **Prasai MJ, George JT, Scott EM.** Molecular clocks, type 2 diabetes and cardiovascular disease. *Diab Vasc Dis Res* 5: 89–95, 2008.
41. **Ramsey KM, Marcheva B, Kohsaka A, Bass J.** The clockwork of metabolism. *Annu Rev Nutr* 27: 219–240, 2007.
42. **Rudic RD, McNamara P, Curtis AM, Boston RC, Panda S, Hogenesch JB, Fitzgerald GA.** BMAL1 and CLOCK, two essential components of the circadian clock, are involved in glucose homeostasis. *PLoS Biol* 2: e377, 2004.
43. **Savignac HM, Finger BC, Pizzo RC, O'Leary OF, Dinan TG, Cryan JF.** Increased sensitivity to the effects of chronic social defeat stress in an innately anxious mouse strain. *Neuroscience* 192: 524–536, 2011.
44. **Schibler U, Sassone-Corsi P.** A web of circadian pacemakers. *Cell* 111: 919–922, 2002.
45. **So AY, Bernal TU, Pillsbury ML, Yamamoto KR, Feldman BJ.** Glucocorticoid regulation of the circadian clock modulates glucose homeostasis. *Proc Natl Acad Sci USA* 106: 17582–17587, 2009.
46. **Solt LA, Wang Y, Banerjee S, Hughes T, Kojetin DJ, Lundasen T, Shin Y, Liu J, Cameron MD, Noel R, Yoo SH, Takahashi JS, Butler AA, Kamenecka TM, Burris TP.** Regulation of circadian behaviour and metabolism by synthetic REV-ERB agonists. *Nature* 29: 62–68, 2012.
47. **Staels B.** When the Clock stops ticking, metabolic syndrome explodes. *Nat Med* 12: 54–55, 2006.
48. **Stratmann M, Schibler U.** Properties, entrainment, and physiological functions of mammalian peripheral oscillators. *J Biol Rhythms* 21: 494–506, 2006.
49. **Tannenbaum BM, Brindley DN, Tannenbaum GS, Dallman MF, McArthur MD, Meaney MJ.** High-fat feeding alters both basal and stress-induced hypothalamic-pituitary-adrenal activity in the rat. *Am J Physiol Endocrinol Metab* 273: E1168–E1177, 1997.
50. **Turek FW, Joshu C, Kohsaka A, Lin E, Ivanova G, McDearmon E, Laposky A, Losee-Olson S, Easton A, Jensen DR, Eckel RH, Takahashi JS, Bass J.** Obesity and metabolic syndrome in circadian Clock mutant mice. *Science* 308: 1043–1045, 2005.
51. **Uno K, Katagiri H, Yamada T, Ishigaki Y, Ogihara T, Imai J, Hasegawa Y, Gao J, Kaneko K, Iwasaki H, Ishihara H, Sasano H, Inukai K, Mizuguchi H, Asano T, Shiota M, Nakazato M, Oka Y.** Neuronal pathway from the liver modulates energy expenditure and systemic insulin sensitivity. *Science* 312: 1656–1659, 2006.
52. **Vrang N, Kristensen P, Tang-Christensen M, Larsen PJ.** Effects of leptin on arcuate pro-opiomelanocortin and cocaine-amphetamine-regulated transcript expression are independent of circulating levels of corticosterone. *J Neuroendocrinol* 14: 880–886, 2002.
53. **Willner P, Muscat R, Papp M.** Chronic mild stress-induced anhedonia: a realistic animal model of depression. *Neurosci Biobehav Rev* 16: 525–534, 1992.
54. **Yamamoto T, Nakahata Y, Tanaka M, Yoshida M, Soma H, Shinohara K, Yasuda A, Mamine T, Takumi T.** Acute physical stress elevates mouse period1 mRNA expression in mouse peripheral tissues via a glucocorticoid-responsive element. *J Biol Chem* 280: 42036–42043, 2005.
55. **Yang X, Downes M, Yu RT, Bookout AL, He W, Straume M, Mangelsdorf DJ, Evans RM.** Nuclear receptor expression links the circadian clock to metabolism. *Cell* 126: 801–810, 2006.
56. **Zhang EE, Liu Y, Dentin R, Pongsawakul PY, Liu AC, Hirota T, Nusinow DA, Sun X, Landais S, Kodama Y, Brenner DA, Montminy M, Kay SA.** Cryptochrome mediates circadian regulation of cAMP signaling and hepatic gluconeogenesis. *Nat Med* 16: 1152–1156, 2010.

Am J Physiol Endocrinol Metab 305: E641–E648, 2013.
First published July 23, 2013; doi:10.1152/ajpendo.00120.2013.

Bach1 deficiency protects pancreatic β-cells from oxidative stress injury

Keiichi Kondo,[1] Yasushi Ishigaki,[1] Junhong Gao,[1] Tetsuya Yamada,[1] Junta Imai,[1] Shojiro Sawada,[1] Akihiko Muto,[2] Yoshitomo Oka,[1] Kazuhiko Igarashi,[2] and Hideki Katagiri[1,3]

[1]*Division of Metabolism and Diabetes, Tohoku University Graduate School of Medicine, Sendai, Japan;* [2]*Department of Biochemistry, Tohoku University Graduate School of Medicine, Sendai, Japan; and* [3]*Japan Science and Technology Agency, CREST, Seiryo-machi, Aoba-ku, Sendai Japan*

Submitted 4 March 2013; accepted in final form 17 July 2013

Kondo K, Ishigaki Y, Gao J, Yamada T, Imai J, Sawada S, Muto A, Oka Y, Igarashi K, Katagiri H. Bach1 deficiency protects pancreatic β-cells from oxidative stress injury. *Am J Physiol Endocrinol Metab* 305: E641–E648, 2013. First published July 23, 2013; doi:10.1152/ajpendo.00120.2013.—BTB and CNC homology 1 (Bach1) is a transcriptional repressor of antioxidative enzymes, such as heme oxygenase-1 (HO-1). Oxidative stress is reportedly involved in insulin secretion impairment and obesity-associated insulin resistance. However, the role of Bach1 in the development of diabetes is unclear. HO-1 expression in the liver, white adipose tissue, and pancreatic islets was markedly upregulated in Bach1-deficient mice. Unexpectedly, glucose and insulin tolerance tests showed no differences in obese wild-type (WT) and obese Bach1-deficient mice after high-fat diet loading for 6 wk, suggesting minimal roles of Bach1 in the development of insulin resistance. In contrast, Bach1 deficiency significantly suppressed alloxan-induced pancreatic insulin content reduction and the resultant glucose elevation. Furthermore, TUNEL-positive cells in pancreatic islets of Bach1-deficient mice were markedly decreased, by 60%, compared with those in WT mice. HO-1 expression in islets was significantly upregulated in alloxan-injected Bach1-deficient mice, whereas expression of other antioxidative enzymes, e.g., catalase, superoxide dismutase, and glutathione peroxidase, was not changed by either alloxan administration or Bach1 deficiency. Our results suggest that Bach1 deficiency protects pancreatic β-cells from oxidative stress-induced apoptosis and that the enhancement of HO-1 expression plays an important role in this protection.

BTB and CNC homology 1; diabetes; insulin; pancreatic β-cells; oxidative stress; apoptosis

TYPE 2 DIABETES IS CAUSED BY COMPLEX INTERACTIONS between insulin resistance in peripheral tissues and impaired insulin secretion from pancreatic β-cells (47). Exacerbation of oxidative stress (19) as well as endoplasmic reticulum stress (50) and hypoxic conditions (44) play key roles in the progressive deterioration of pancreatic β-cells during the development of diabetes. Pancreatic β-cells are a direct target of oxidative stress-mediated effects. Oxidants have been shown to negatively affect mitochondrial action in pancreatic β-cells, leading to impaired insulin secretion (25). However, pancreatic islets have very low intrinsic antioxidant enzyme capacity compared with other tissues (8), making islets more vulnerable to oxidative stress than other tissues. Therefore, both reducing oxidative stress and enhancing the antioxidative system may be beneficial strategies for preserving β-cell function.

On the other hand, oxidative stress also plays a crucial role in obesity-associated insulin resistance (46). Obesity increases reactive oxygen species (ROS) production via NADPH oxidase activation in adipose tissue (6). Excessive generation of ROS impairs glucose uptake into muscle (24) and adipose tissues (37) and is involved in the pathogenesis of hepatic steatosis (36), collectively leading to systemic insulin resistance. In addition, oxidative stress directly causes dysregulated production of adipocytokines such as adiponectin, plasminogen activator inhibitor-1, and monocyte chemotactic protein-1 (6), resulting in deterioration of insulin sensitivity. Thus, oxidative stress is closely related to obesity-associated insulin resistance and thereby contributes to the vicious cycle leading to the development of metabolic syndrome.

Transcription factor BTB and CNC homology 1 (Bach1) is a basic region leucine zipper transcription factor that suppresses oxidative stress responses (33). Bach1 forms a heterodimer with small Maf oncoproteins and binds to Maf recognition elements (MAREs) on the genome, thereby inhibiting transcription of oxidative stress-responsive genes, including heme oxygenase-1 (HO-1) (30), NAD(P)H quinone oxidoreductase 1 (2), and thioredoxin reductase 1 (10). Expression of these genes is very low under normal conditions due the dominant binding of Bach1 to MAREs. Once cells are subjected to oxidative stress, Bach1 loses its DNA-binding activity and is exported out of the nuclei. Instead of Bach1, NF-E2-related factor (Nrf) 2, another basic region leucine zipper transcription factor, rapidly accesses and binds to MAREs, resulting in activation of the transcription of oxidative stress-responsive genes (38). Indeed, in Bach1-deficient mice, HO-1 is constitutively expressed at higher levels in many tissues, indicating that Bach1 plays a major role in negative regulation of HO-1 expression (39).

It is particularly noteworthy that pharmacological enhancement of the HO-1 pathway reportedly exerts protective effects against the development of diabetes via several mechanisms (1). Upregulation of HO-1 protects pancreatic β-cells from oxidative injury induced by high glucose concentrations, resulting in preservation of insulin secretion in vitro (48). Administration of an HO-1 inducer decreases pancreatic superoxide contents, which in turn results in suppression of β-cell loss in nonobese diabetic (NOD) mice, a well-established model of type 1 diabetes (22). In addition to its effects on insulin secretion, HO-1 induction suppresses fat accumulation (21, 29) and insulin resistance (28) in obese rodents.

Previous studies have revealed Bach1-deficient mice to be protected from a wide range of pathological conditions, including myocardial infarction (51), atherosclerosis (45), steatohepatitis (12), intestinal injury (9), lung injury (43), keratinocyte differentiation (31), and neural tissue damage (16). Thus, Bach1 deficiency may protect mice against oxidative tissue damage. These findings prompted us to hypothesize that ablation of Bach1 would be a promising approach toward prevent-

Address for reprint requests and other correspondence: Y. Ishigaki, Div. of Metabolism and Diabetes, Tohoku Univ. Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan (e-mail: yishigaki@med.tohoku.ac.jp).

0193-1849/13 Copyright © 2013 the American Physiological Society

ing the development of insulin resistance, insulin secretion impairment, and ultimately diabetes. Therefore, in this study, we examined the effects of Bach1 deficiency on glucose metabolism in two different murine models of diabetes, i.e., alloxan-induced pancreatic β-cell loss and high-fat diet (HFD)-induced insulin resistance.

METHODS

Animals. Animal studies were conducted in accordance with the institutional guidelines for animal experiments at The Center for Laboratory Animal Research, Tohoku University, which approved the experiments. Bach1-deficient mice were backcrossed with C57BL/6J mice (39). Wild-type (WT) and Bach1-deficient mice were kept on a 12:12-h light-dark cycle with free access to food and sterile water. For the purpose of oxidative stress loading of pancreatic β-cells, a 50 mg/kg dose of alloxan (2,4,5,6-tetraoxypyrimidine; Sigma-Aldrich, St. Louis, MO) was injected intravenously into fasted 8-wk-old mice (49). In detail, a 10 mg/ml alloxan-citrate solution was prepared with ice-cold 0.05 M citrate buffer (pH 4.3) immediately prior to injection. In the control group of mice, the same volume of citrate buffer was injected intravenously. Mice with HFD-induced obesity were obtained by 18-wk feeding of a HFD (32% safflower oil, 33.1% casein, 17.6% sucrose, and 5.6% cellulose) (14) beginning at 6 wk of age.

Analysis of glucose metabolism. Blood glucose was assayed with Antsense-III (Horiba Industry, Kyoto, Japan). Plasma insulin concentrations were determined using an ELISA kit (Morinaga, Tokyo, Japan). Glucose tolerance tests were performed on fasted (10 h, daytime) mice. The mice were given glucose (1 or 2 g/kg of body wt) intraperitoneally, followed by measurement of blood glucose levels. Insulin tolerance tests were performed on ad libitum-fed mice. The mice were injected intraperitoneally with human regular insulin (0.5 or 0.75 U/kg body wt; Eli Lilly, Kobe, Japan), followed by measurement of blood glucose levels (7).

Quantitative RT-PCR-based gene expression. Quantitative RT-PCR was performed as described previously (13). The relative amount of mRNA was calculated, employing GAPDH mRNA as the invariant control. The oligonucleotide primers are presented in Table 1.

Histological analysis. The pancreases from WT and Bach1-deficient mice were fixed with 10% formalin, embedded in paraffin, and sectioned. Sections were stained with hematoxylin and eosin (H & E).

Laser microdissection. Coronal cryostat sections (10 μm) of the pancreas were placed on PEN-coated slides (Leica Microsystems, Wetzler, Germany). Laser microdissection was carried out on a Leica AS LMD (Leica Microsystems). Immediately after microdissection of each islet, the samples were stored at −80°C until RNA purification (15).

Table 1. *Oligonucleotide primers used in quantitative RT-PCR*

Primer	Sequence
HO-1 forward	5′-ATGGCGTCACTTCGTCAGAG
HO-1 reverse	5′-AATTCCCACTGCCACTGTTG
GAPDH forward	5′-TGAAGGTCGGTGTGAACG
GAPDH reverse	5′-CCATTCTCGGCCTTGACT
Catalase forward	5′-AGGTGTTGAACGAGGAGGA
Catalase reverse	5′-CTCAGCGTTGTACTTGTCCA
SOD forward	5′-GGTCGCTTACAGATTGCT
SOD reverse	5′-CTCCCAGTTGATTACATTCC
GPx forward	5′-TGCAGAAGCGTCTGGGACCT
GPx reverse	5′-GGTCGGACGTACTTGAGGGA
p22 phox forward	5′-CCATTGCCAGTGTGATCTAT
p22 phox reverse	5′-GTTGGTAGGTGGTTGCTTGA
gp91phox forward	5′-TGTGCACCATGATGAGGA
gp91phox reverse	5′-GTTGGAGATGCTCTGTTTACTG

HO-1, heme oxygenase; SOD, superoxide dismutase; GPx, glutathione peroxidase.

Apoptosis detection. At 24 h after alloxan injection, DNA fragmentation associated with apoptosis was detected in situ by the addition of nucleotides to free 3′-hydroxyl groups in DNA. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed using an in situ cell death detection kit (Roche, Mannheim, Germany). The results are expressed as the number of TUNEL-positive cells per islet, as reported previously.

Pancreatic insulin contents. Pancreases were suspended in cold acid ethanol (1.5% HCl in 75% ethanol), minced with scissors, and left at −20°C for 48 h, with sonication every 24 h. Insulin contents in the acid ethanol supernatant were determined with an ELISA kit (Morinaga) (41).

Statistical analysis. All statistical analyses were performed with the Statistical Package for the Social Sciences version 15.0 (SPSS Japan, Tokyo, Japan). All data were tested for normality by the Kolmogorov-Smirnov test. When data were distributed normally, the statistical significance of differences was assessed using the unpaired *t*-test and one-way ANOVA, followed by Tukey post hoc analyses. When data were not distributed normally, the statistical significance of differences was judged based on *P* values, using the Mann-Whitney *U*-test.

RESULTS

Bach1 deficiency exerted minimal effects on glucose metabolism under standard feeding conditions. First, we measured body weights and blood glucose levels until 12 wk of age, followed by analysis of glucose metabolism under standard feeding conditions. Body weights were similarly increased and fasting blood glucose levels were also similar in WT and Bach1-deficient mice (Fig. 1*A*). In addition, neither blood pressure nor plasma insulin differed significantly between the two groups at 12 wk of age (Fig. 1*B*). Furthermore, glucose and insulin tolerance tests revealed that Bach1 deficiency affected neither systemic glucose tolerance nor insulin sensitivity (Fig. 1, *C* and *D*). Histological findings with H & E staining revealed no apparent differences in pancreatic islet morphology between WT and Bach1-deficient mice (Fig. 1*E*). In addition, pancreatic insulin contents were also similar in Bach1-deficient and WT mice (Fig. 1*F*). Thus, Bach1 deficiency had no significant effects on systemic glucose metabolism under standard feeding conditions.

In Bach1-deficient mice, HO-1 is constitutively expressed at very high levels in many tissues, such as the liver, lungs, and heart (39). We next examined expression of HO-1 in organs/tissues responsible for glucose metabolism. As shown in Fig. 1*G*, HO-1 expression was markedly increased not only in the liver but also in pancreatic islets and white adipose tissue.

Bach1 deficiency minimally impacts obesity and insulin resistance. Pharmacological HO-1 upregulation reportedly prevents the development of diabetes by both maintaining pancreatic β-cell function (48) and decreasing adiposity and insulin resistance (21). Therefore, we next examined metabolic effects of Bach1 deficiency on insulin resistance in models of HFD-induced obesity. First, WT and Bach1-deficient mice were started on a HFD at 18 wk of age. In Bach1-deficient mice, body weights were similarly increased and blood glucose was elevated during the 6 wk of HFD feeding compared with WT mice (Fig. 2*A*). Furthermore, neither glucose tolerance nor systemic insulin sensitivity (Fig. 2*B*) differed between WT and Bach1-deficient mice after 6 wk of HFD loading. Fasting insulin levels were similarly increased during the 18 wk of HFD feeding in the two groups (Fig. 2*C*). These findings

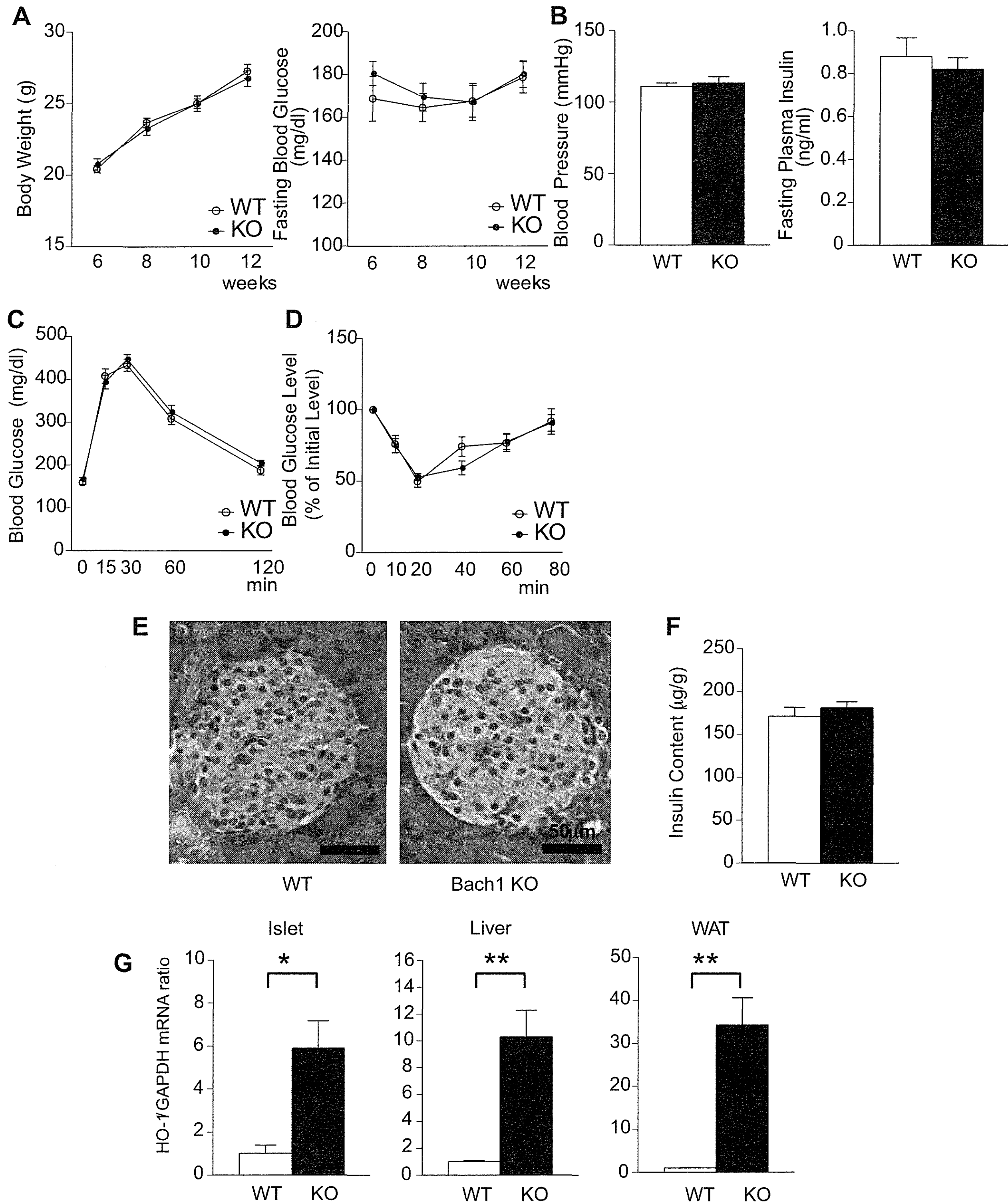


Fig. 1. Transcription factor BTB and CNC homology 1 (Bach1) deficiency exerted minimal effects on glucose metabolism under standard feeding conditions. *A*: body weight and fasting blood glucose of wild-type (WT) (○; $n = 12$) and Bach1-deficient mice (KO) (●; $n = 12$) from 6 to 12 wk of age. *B*: blood pressure at 8 wk of age in WT (open bar; $n = 16$) and Bach1-deficient mice (black bar; $n = 8$). Fasting plasma insulin at 12 wk of age in WT (open bar; $n = 12$) and Bach1-deficient mice (black bar; $n = 12$). *C*: intraperitoneal glucose (2 mg/kg) tolerance tests at 10 wk of age in WT (○; $n = 7$) and Bach1-deficient mice (●; $n = 7$). *D*: insulin (0.5 U/kg) tolerance tests at 12 wk of age in WT (○; $n = 9$) and Bach1-deficient mice (●; $n = 8$). *E*: hematoxylin and eosin staining of pancreatic islets of WT (*left*) and Bach1-deficient mice (*right*) at 12 wk of age. *F*: insulin contents adjusted by pancreatic weights of WT (open bar; $n = 8$) and Bach1-deficient mice (black bar; $n = 8$) at 12 wk of age. *G*: quantitative RT-PCR for heme oxygenase-1 (HO-1) of pancreatic islets at 8 wk of age ($n = 4$ each) and in the liver and white adipose tissue (WAT) at 12 wk of age ($n = 8$ each). WT (open bars) and Bach1-deficient mice (black bars). Data are presented as means ± SE. $^*P < 0.05$ and $^{**}P < 0.01$, assessed by unpaired *t*-test.

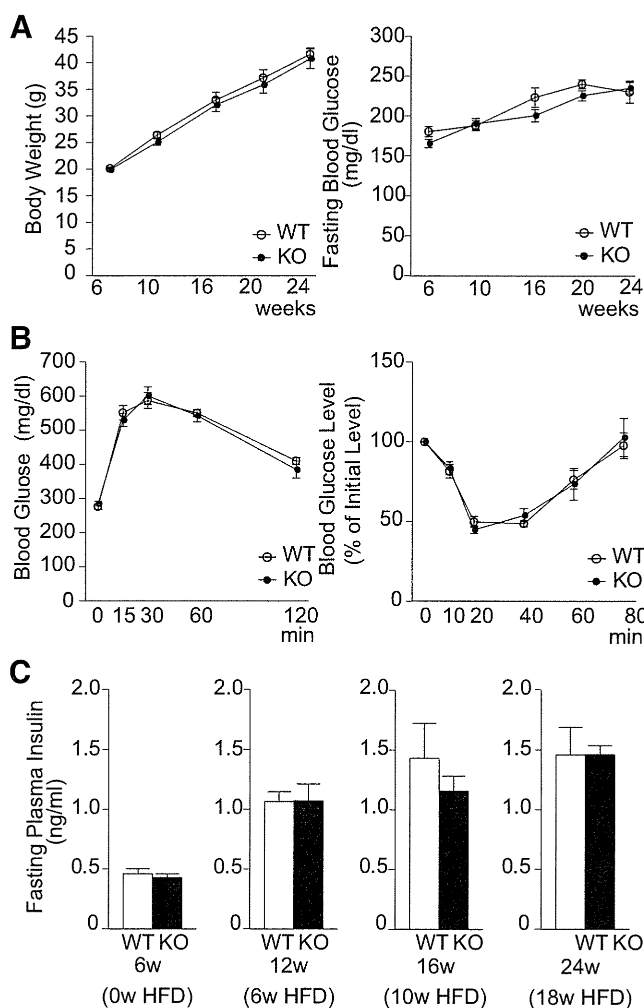


Fig. 2. Bach1 deficiency minimally impacts obesity and insulin resistance. WT and Bach1-deficient mice were fed a HFD starting at 6 wk of age. *A*: body weights and fasting blood glucose values of WT (○; n = 6–8) and KO (●; n = 8–12) mice from 6 to 24 wk of age (n = 8 each). *B*: intraperitoneal glucose (2 mg/kg) tolerance tests at 12 wk in WT (○; n = 8) and KO mice (●; n = 7) and insulin (0.75 U/kg) tolerance tests at 13 wk of age in WT (○; n = 5) and KO mice (ccirc; n = 5). *C*: fasting plasma insulin from 6 to 24 wk of age in WT (open bars; n = 6–8) and KO mice (black bars; n = 8–12). Data are presented as means ± SE.

suggest minimal roles of Bach1 in the development of obesity and insulin resistance.

Bach1 deficiency suppressed alloxan-induced pancreatic β-cell injury. Next, to examine the effects of Bach1 deficiency on pancreatic β-cell injury induced by oxidative stress, we administered alloxan to Bach1-deficient mice at 8 wk of age. Alloxan is an oxidative stress agent that is relatively specific to pancreatic β-cells, and its administration is widely accepted as a model of diabetes caused by β-cell loss (18). When a 50 mg/kg dose of alloxan was administered to WT mice intravenously, fasting blood glucose gradually rose, reaching ~200 mg/dl on *day 6* after alloxan administration was started (Fig. 3*A*). In contrast, fasting blood glucose levels were significantly lower in Bach1-deficient than in WT mice. In addition, glucose tolerance tests on *day 6* after alloxan administration was started revealed that glucose elevation after a glucose load was consistently suppressed in Bach1-deficient mice compared with WT mice (Fig. 3*B*). Furthermore, pancreatic insulin contents were significantly higher, approximately twofold, in Bach1-deficient than in WT mice (Fig. 3*C*). Thus, Bach1 deficiency protected mice from alloxan-induced injury of pancreatic β-cells.

Oxidative stress is one of the most important causes of pancreatic β-cell death during the development of diabetes (19), and many studies have shown relationships between β-cell loss and ROS accumulation-induced pancreatic β-cell apoptosis (35). Therefore, to determine the effect of Bach1 on alloxan-induced β-cell apoptosis, we measured the number of TUNEL-positive cells in pancreatic islets 24 h after alloxan administration. At this time point, pancreatic islet sizes were similar in the two groups of mice (Fig. 4, *A*, *C*, *E*, *G*, and *J*). TUNEL-positive pancreatic β-cells were quite rare in both types of mice in the absence of alloxan injection (Fig. 4, *B* and *F*). Although TUNEL-positive cells were clearly detected in islets of both types of alloxan-treated mice (Fig. 4, *D* and *H*), the number of TUNEL-positive cells in Bach1-deficient mice was markedly decreased, being 60% less than in WT mice (Fig. 4*I*). These findings indicate that Bach1 deficiency protected pancreatic β-cells from oxidative stress-induced apoptosis.

In addition, using the laser microdissection procedure, we evaluated mRNA expression of genes related to oxidative stress. Alloxan administration-induced HO-1 upregulation in islets was enhanced in Bach1-deficient mice (Fig. 4*K*). In contrast, expression of other antioxidative enzymes, e.g., catalase, superoxide dismutase, and glutathione peroxidase, showed no significant changes in response to either alloxan administration or Bach1 deficiency (Fig. 4*K*). These findings, taken together, suggest that enhancement of HO-1 expression plays a major role in the antioxidant effect of Bach1 deficiency. In addition, expression of p22phox and gp91phox, both of which are subunits of NADPH oxidase, was decreased in pancreatic islets of alloxan-treated Bach1-deficient mice compared with those of alloxan-treated WT mice (Fig. 4*L*). These findings suggest that decreased ROS production is also involved in pancreatic β-cell protection in Bach1-deficient mice.

DISCUSSION

The present study showed the effect of Bach1 deficiency on β-cell protection against acute loading of a chemical oxidant, alloxan, that reportedly induces apoptosis in pancreatic β-cells. Bach1 deficiency preserved insulin content and suppressed apoptosis of pancreatic islet cells, resulting in amelioration of alloxan-induced hyperglycemia. Thus, Bach1 in pancreatic β-cells is involved in oxidative stress-induced β-cell dysfunction and apoptosis, which may underlie the development of both type 1 and type 2 diabetes. In the present study, Bach1 deficiency decreased the alloxan-induced apoptosis of pancreatic islet cells. This protective effect against oxidative stress resulted in suppression of hyperglycemia in Bach1-deficient mice. These results indicate that Bach1 deficiency-induced HO-1 upregulation prevented pancreatic β-cell deterioration induced by oxidative stress.

A putative mechanism underlying Bach1 deficiency-induced protection of pancreatic β-cells is HO-1 upregulation. HO-1 has been identified as a ubiquitous stress protein upregulated in many cell types by various stimulants, including hemolysis, inflammatory cytokines, oxidative stress, and heat shock (26).

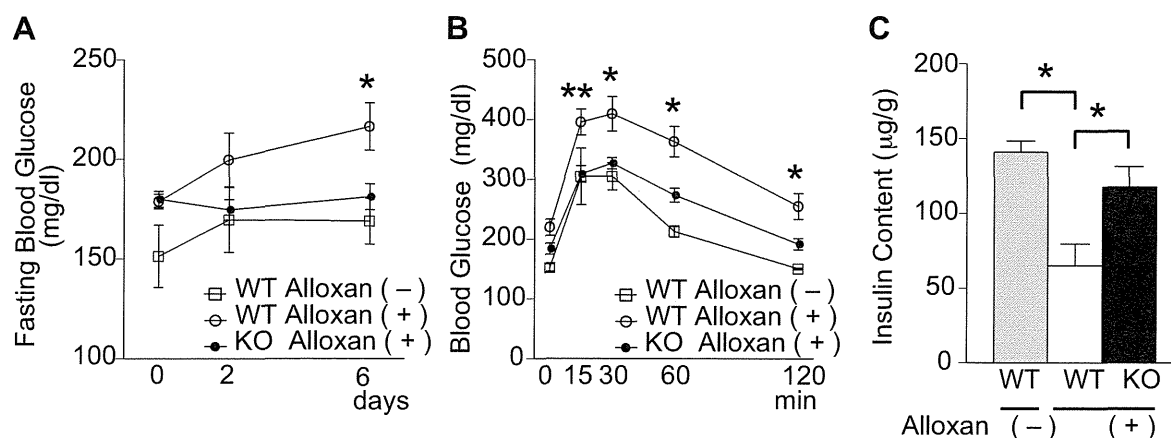


Fig. 3. Bach1 deficiency suppressed alloxan-induced hyperglycemia and reduced pancreatic insulin contents. Alloxan or citrate buffer (control) was injected intravenously into WT and KO mice at 8 wk of age. *A*: fasting blood glucose 1 h before and 2 and 6 days after 50 mg/kg alloxan injection. WT (○; n = 30), KO (●; n = 32), and WT control mice (□; n = 5). *B*: intraperitoneal glucose (1 mg/kg) tolerance tests 6 days after 50 mg/kg alloxan injection into WT (○; n = 19) and KO (●; n = 20) as well as WT control (□; n = 2) mice. *C*: insulin content 7 days after 50 mg/kg alloxan injection into WT (open bar; n = 9) and KO (black bar; n = 7) as well as WT control mice (gray bar; n = 3). Data are presented as means ± SE. $^{*}P < 0.05$ and $^{**}P < 0.01$, assessed by 1-way ANOVA followed by Tukey post hoc analyses (asterisks in *A* and *B* show the significance between alloxan-injected WT and alloxan-injected Bach1-deficient mice).

HO-1 expression is induced by the Nrf2 system, which is suppressed in the absence of oxidative stress stimuli (17). HO-1 is the rate-limiting enzyme in the heme degradative pathway that catalyzes the oxidation of heme into biliverdin, carbon monoxide (CO), and free iron. CO as well as biliverdin and its metabolite bilirubin have antioxidant activities in vivo (32). Thus, HO-1 plays a crucial role in protection from oxidative stress (5). Induction or transgenic overexpression of HO-1 reportedly renders pancreatic islets resistant to apoptosis in islet transplantation models (34) and NOD mice (11). Administration of an HO-1 inducer decreases pancreatic superoxide contents, leading to suppression of β-cell reduction in NOD mice (22). In the present study, we observed that Bach1 deficiency induced constitutive expression of HO-1 in pancreatic islets. In addition, alloxan-induced HO-1 upregulation was markedly promoted in Bach1-deficient mice. In contrast, other antioxidative enzymes were not significantly upregulated in pancreatic islets by either Bach1 deficiency or alloxan administration. Taken together, these observations suggest that HO-1 plays an important role in the antioxidant effects of Bach1 deficiency. Since p22phox expression is reportedly upregulated by ROS (3), the observed p22phox downregulation suggests decreased ROS levels in pancreatic islets of Bach1-deficient mice. Thus, antioxidant effects induced by Bach1 deficiency may further suppress ROS production. In addition, expression of gp91phox, which reportedly plays a major role in ROS production in pancreatic β-cells (23), was downregulated in pancreatic islets of Bach1-deficient mice. Although the mechanism whereby Bach1 deficiency suppresses gp91phox expression is unclear, suppression of ROS production by downregulation of NADPH oxidase may also contribute to the pancreatic β-cell protection mediated by Bach1 deficiency. Thus, Bach1 inhibition by pharmacological strategies is a potential therapeutic target for pancreatic β-cell protection against oxidative stress, although pharmacological reagents that suppress Bach1 expression and/or function have not yet been developed.

Chemical HO-1 inducers reportedly improve insulin resistance in murine obesity models (21, 28, 29). Unexpectedly, however, Bach1 deficiency had no impact on either insulin resistance or obesity even after long-term HFD loading. Thus, in contrast to acute induction of HO-1, congenital prolonged high expression of HO-1 might have less impact on insulin sensitivity. HO-1 is an inducible protein and is thought to act as a defense mechanism against acute oxidative injuries such as inflammation, ischemia, and radiation. Because the cytoprotective function of HO-1 is manifested by reaction products such as bilirubin and CO, amounts of the substrates heme and/or oxygen could be the limiting factor in HO-1 function (42). It is possible that depletion of free heme (i.e., not bound to proteins) due to constitutive HO-1 expression would cancel the effects on insulin resistance in Bach1-deficient mice. This may also explain the differential effects of Bach1 deficiency on acute injury of β-cells and chronic development of insulin resistance. In addition, differences in HO-1 expression levels may contribute to the diversity of effects in various tissues/organs. According to an HO-1 transgenic study, low levels (2- to 5-fold) of HO-1 overexpression are protective, whereas moderate levels (10- to 15-fold) do not modify the cell injury caused by hyperoxia, and high-level (≥15-fold) overexpression actually exacerbates the damage (40). In the present study, the HO-1 expression levels in Bach1-deficient mice were increased 12-fold in the liver and 30-fold in white adipose tissue, whereas the increase was sixfold in pancreatic islets. Thus, constant and markedly elevated HO-1 expression may deplete the necessary heme substrate, resulting in minimal effects of Bach1 deficiency on metabolism. It has been reported recently that sustained expression of HO-1 in cultured cells leads to profound changes to cellular iron homeostasis (20). Such adaptive changes may also suppress the protective function of HO-1. Alternatively, a pathway(s) other than HO-1 may exacerbate HFD-induced insulin resistance in Bach1-deficient mice, thereby cancelling out the beneficial effects of HO-1. For instance, Bach1 was reported to inhibit p53 transcriptional activity (4). Overexpression of p53 in adipose tissue reportedly causes insulin resistance via elevated inflammatory responses (27). Collectively, chronic HO-1 elevation showed minimal effects on the development of insulin resistance induced by high-fat feeding. Thus, the proper levels and duration

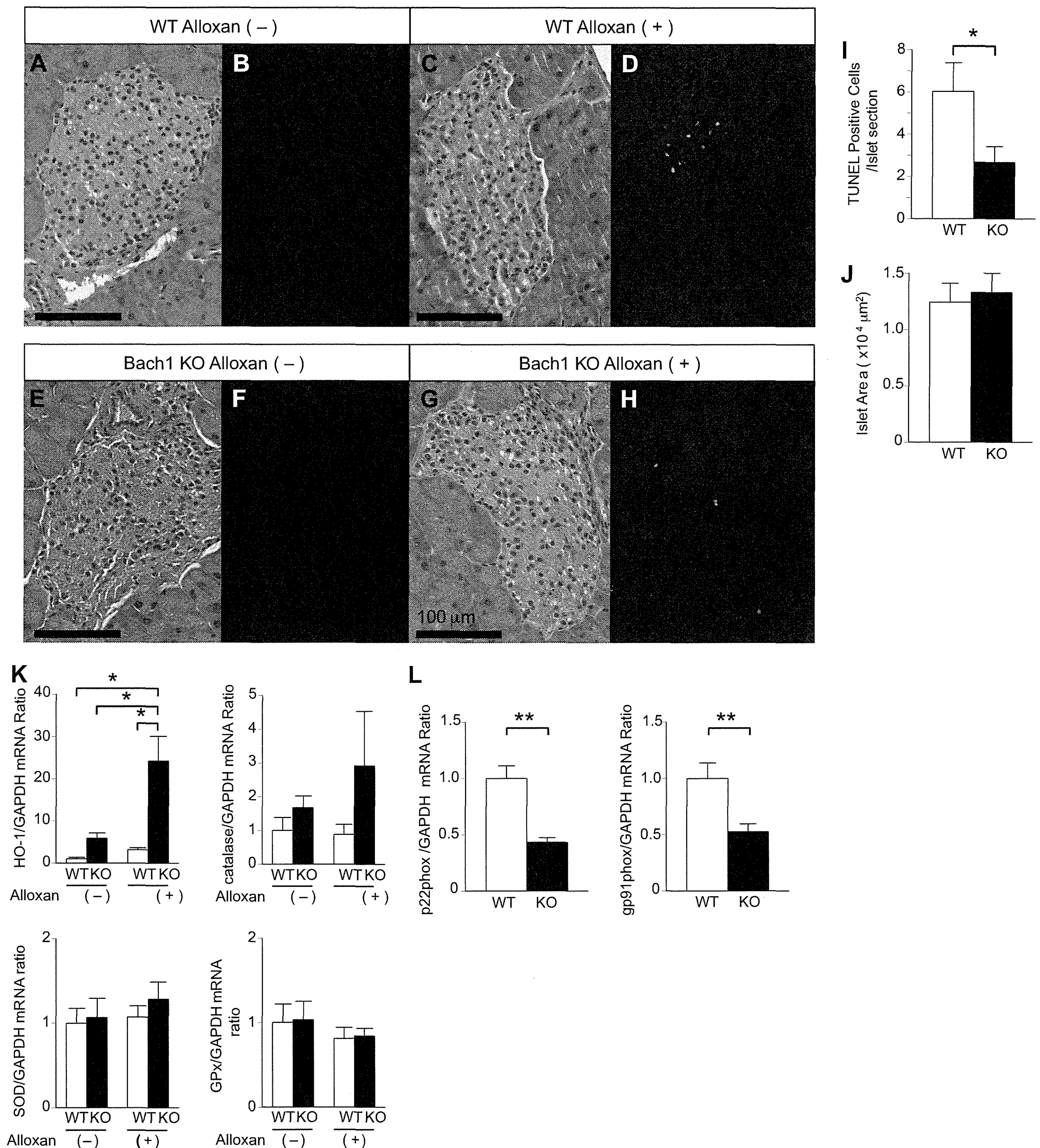


Fig. 4. Suppression of apoptosis of pancreatic islet cells and upregulation of HO-1 expression in alloxan-injected KO mice. Alloxan or citrate buffer (control) was injected into 8-wk-old WT and KO mice. *A–H*: hematoxylin and eosin staining and TUNEL staining of pancreases taken 24 h after injection of 50 mg/kg alloxan. Paired adjacent slices were used for hematoxylin and eosin and TUNEL staining. *A* and *B*: WT control. *C* and *D*: alloxan-injected WT. *E* and *F*: KO control. *G* and *H*: alloxan-injected KO mice. *I* and *J*: nos. of TUNEL-positive cells/islet slice and mean islet areas in alloxan-injected mice. The average islet count was 12/slice for WT and 14/slice for Bach1-deficient mice. WT (open bars; $n = 36$) and KO islets (black bars; $n = 42$) were measured. *K*: quantitative RT-PCR for HO-1, catalase, superoxide dismutase (SOD), and glutathione peroxidase (GPx) of pancreatic islets 2 h after alloxan or citrate buffer injection. WT control (*left* open bars; $n = 4$), KO control (*left* black bars; $n = 4$), alloxan-injected WT (*right* open bars; $n = 5$), and alloxan-injected KO mice (*right* black bars; $n = 6$) were studied. *L*: quantitative RT-PCR for p22 phox and gp91phox of pancreatic islets 2 h after alloxan. Alloxan-injected WT (open bar; $n = 4$) and alloxan-injected KO mice (black bar; $n = 6$) were studied. Data are presented as means ± SE. *$P < 0.05$, assessed by Mann-Whitney *U*-test (*I*) and 1-way ANOVA, followed by Tukey post hoc analyses (*K*); **$P < 0.01$, assessed by unpaired *t*-test (*L*).

of HO-1 induction are important for the protection of cells from oxidative stress. Application of a chemical HO-1 inducer clinically, especially for insulin resistance, would require close attention to the levels and duration of HO-1 expression.

In conclusion, our results indicate Bach1 deficiency to have preventive effects against oxidative stress-induced β-cell injury. In addition to the numerous beneficial effects observed in Bach1-deficient mice, these findings provide clinically applicable evidence that Bach1 deficiency protects against the development of diabetes, making it a possible treatment target for diabetes prevention.

ACKNOWLEDGMENTS

We thank J. Fushimi, R. Tomono, M. Hoshi, and T. Takasugi for technical support.

GRANTS

This work was supported by Grants-in-Aid for Scientific Research (B2, 15390282) to H. Katagiri and (21591122) Y. Ishigaki from the Japan Society for the Promotion of Science, a Grant-in-Aid for Scientific Research on Innovative Areas (to H. Katagiri), and the Global-COE (Y. Oka and H. Katagiri) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

DISCLOSURES

No conlficts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

K.K., J.G., and S.S. performed the experiments; K.K., J.G., and S.S. analyzed the data; K.K. prepared the figures; Y.I., Y.O., K.I., and H.K. contributed to the conception and design of the research; Y.I. and H.K. drafted the manuscript; T.Y., J.I., A.M., and H.K. interpreted the results of the experiments.

REFERENCES

1. **Abraham NG, Kappas A.** Pharmacological and clinical aspects of heme oxygenase. *Pharmacol Rev* 60: 79–127, 2008.
2. **Dhakshinamoorthy S, Jain AK, Bloom DA, Jaiswal AK.** Bach1 competes with Nrf2 leading to negative regulation of the antioxidant response element (ARE)-mediated NAD(P)H:quinone oxidoreductase 1 gene expression and induction in response to antioxidants. *J Biol Chem* 280: 16891–16900, 2005.
3. **Djordjevic T, Pogrebniak A, BelAiba RS, Bonello S, Wotzlaw C, Acker H, Hess J, Gorlach A.** The expression of the NADPH oxidase subunit p22phox is regulated by a redox-sensitive pathway in endothelial cells. *Free Radic Biol Med* 38: 616–630, 2005.
4. **Dohi Y, Ikura T, Hoshikawa Y, Katoh Y, Ota K, Nakanome A, Muto A, Omura S, Ohta T, Ito A, Yoshida M, Noda T, Igarashi K.** Bach1 inhibits oxidative stress-induced cellular senescence by impeding p53 function on chromatin. *Nat Struct Mol Biol* 15: 1246–1254, 2008.
5. **Ferris CD, Jaffrey SR, Sawa A, Takahashi M, Brady SD, Barrow RK, Tysoe SA, Wolosker H, Baranano DE, Dore S, Poss KD, Snyder SH.** Haem oxygenase-1 prevents cell death by regulating cellular iron. *Nat Cell Biol* 1: 152–157, 1999.
6. **Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Nakayama O, Makishima M, Matsuda M, Shimomura I.** Increased oxidative stress in obesity and its impact on metabolic syndrome. *J Clin Invest* 114: 1752–1761, 2004.
7. **Gao J, Katagiri H, Ishigaki Y, Yamada T, Ogihara T, Imai J, Uno K, Hasegawa Y, Kanzaki M, Yamamoto TT, Ishibashi S, Oka Y.** Involvement of apolipoprotein E in excess fat accumulation and insulin resistance. *Diabetes* 56: 24–33, 2007.
8. **Grankvist K, Marklund SL, Taljedal IB.** CuZn-superoxide dismutase, Mn-superoxide dismutase, catalase and glutathione peroxidase in pancreatic islets and other tissues in the mouse. *Biochem J* 199: 393–398, 1981.
9. **Harusato A, Naito Y, Takagi T, Uchiyama K, Mizushima K, Hirai Y, Yamada S, Tuji T, Yoriki H, Horie R, Inoue K, Fukumoto K, Handa O, Ishikawa T, Kokura S, Minamiyama Y, Ichikawa H, Muto A, Igarashi K, Yoshikawa T.** Suppression of indomethacin-induced apoptosis in the small intestine due to Bach1 deficiency. *Free Radic Res* 45: 717–727, 2011.
10. **Hintze KJ, Katoh Y, Igarashi K, Theil EC.** Bach1 repression of ferritin and thioredoxin reductase1 is heme-sensitive in cells and in vitro and coordinates expression with heme oxygenase1, beta-globin, and NADP(H) quinone (oxido) reductase1. *J Biol Chem* 282: 34365–34371, 2007.
11. **Huang SH, Chu CH, Yu JC, Chuang WC, Lin GJ, Chen PL, Chou FC, Chau LY, Sytwu HK.** Transgenic expression of haem oxygenase-1 in pancreatic beta cells protects non-obese mice used as a model of diabetes from autoimmune destruction and prolongs graft survival following islet transplantation. *Diabetologia* 53: 2389–2400, 2010.
12. **Inoue M, Tazuma S, Kanno K, Hyogo H, Igarashi K, Chayama K.** Bach1 gene ablation reduces steatohepatitis in mouse MCD diet model. *J Clin Biochem Nutr* 48: 161–166, 2011.
13. **Ishigaki Y, Katagiri H, Gao J, Yamada T, Imai J, Uno K, Hasegawa Y, Kaneko K, Ogihara T, Ishihara H, Sato Y, Takikawa K, Nishimichi N, Matsuda H, Sawamura T, Oka Y.** Impact of plasma oxidized low-density lipoprotein removal on atherosclerosis. *Circulation* 118: 75–83, 2008.
14. **Ishigaki Y, Katagiri H, Yamada T, Ogihara T, Imai J, Uno K, Hasegawa Y, Gao J, Ishihara H, Shimosegawa T, Sakoda H, Asano T, Oka Y.** Dissipating excess energy stored in the liver is a potential treatment strategy for diabetes associated with obesity. *Diabetes* 54: 322–332, 2005.
15. **Kaneko K, Yamada T, Tsukita S, Takahashi K, Ishigaki Y, Oka Y, Katagiri H.** Obesity alters circadian expressions of molecular clock genes in the brainstem. *Brain Res* 1263: 58–68, 2009.
16. **Kanno H, Ozawa H, Dohi Y, Sekiguchi A, Igarashi K, Itoi E.** Genetic ablation of transcription repressor Bach1 reduces neural tissue damage and improves locomotor function after spinal cord injury in mice. *J Neurotrauma* 26: 31–39, 2009.
17. **Kobayashi M, Yamamoto M.** Molecular mechanisms activating the Nrf2-Keap1 pathway of antioxidant gene regulation. *Antioxid Redox Signal* 7: 385–394, 2005.
18. **Lenzen S.** The mechanisms of alloxan- and streptozotocin-induced diabetes. *Diabetologia* 51: 216–226, 2008.
19. **Lenzen S.** Oxidative stress: the vulnerable beta-cell. *Biochem Soc Trans* 36: 343–347, 2008.
20. **Li C, Lonn ME, Xu X, Maghzal GJ, Frazer DM, Thomas SR, Halliwell B, Richardson DR, Anderson GJ, Stocker R.** Sustained expression of heme oxygenase-1 alters iron homeostasis in nonerythroid cells. *Free Radic Biol Med* 53: 366–374, 2012.
21. **Li M, Kim DH, Tsenovoy PL, Peterson SJ, Rezzani R, Rodella LF, Aronow WS, Ikehara S, Abraham NG.** Treatment of obese diabetic mice with a heme oxygenase inducer reduces visceral and subcutaneous adiposity, increases adiponectin levels, and improves insulin sensitivity and glucose tolerance. *Diabetes* 57: 1526–1535, 2008.
22. **Li M, Peterson S, Husney D, Inaba M, Guo K, Kappas A, Ikehara S, Abraham NG.** Long-lasting expression of HO-1 delays progression of type I diabetes in NOD mice. *Cell Cycle* 6: 567–571, 2007.
23. **Li N, Li B, Brun T, Deffert-Delbouille C, Mahiout Z, Daali Y, Ma XJ, Krause KH, Maechler P.** NADPH oxidase NOX2 defines a new antagonistic role for reactive oxygen species and cAMP/PKA in the regulation of insulin secretion. *Diabetes* 61: 2842–2850, 2012.
24. **Maddux BA, See W, Lawrence JC, Jr, Goldfine AL, Goldfine ID, Evans JL.** Protection against oxidative stress-induced insulin resistance in rat L6 muscle cells by mircomolar concentrations of alpha-lipoic acid. *Diabetes* 50: 404–410, 2001.
25. **Maechler P, Jornot L, Wollheim CB.** Hydrogen peroxide alters mitochondrial activation and insulin secretion in pancreatic beta cells. *J Biol Chem* 274: 27905–27913, 1999.
26. **Maines MD.** Heme oxygenase: function, multiplicity, regulatory mechanisms, and clinical applications. *FASEB J* 2: 2557–2568, 1988.
27. **Minamino T, Orimo M, Shimizu I, Kunieda T, Yokoyama M, Ito T, Nojima A, Nabetani A, Oike Y, Matsubara H, Ishikawa F, Komuro I.** A crucial role for adipose tissue p53 in the regulation of insulin resistance. *Nat Med* 15: 1082–1087, 2009.
28. **Ndisang JF, Lane N, Jadhav A.** Upregulation of the heme oxygenase system ameliorates postprandial and fasting hyperglycemia in type 2 diabetes. *Am J Physiol Endocrinol Metab* 296: E1029–E1041, 2009.
29. **Nicolai A, Li M, Kim DH, Peterson SJ, Vanella L, Positano V, Gastaldelli A, Rezzani R, Rodella LF, Drummond G, Kusmic C, L'Abbate A, Kappas A, Abraham NG.** Heme oxygenase-1 induction

remodels adipose tissue and improves insulin sensitivity in obesity-induced diabetic rats. *Hypertension* 53: 508–515, 2009.
30. **Ogawa K, Sun J, Taketani S, Nakajima O, Nishitani C, Sassa S, Hayashi N, Yamamoto M, Shibahara S, Fujita H, Igarashi K.** Heme mediates derepression of Maf recognition element through direct binding to transcription repressor Bach1. *EMBO J* 20: 2835–2843, 2001.
31. **Okada S, Muto A, Ogawa E, Nakanome A, Katoh Y, Ikawa S, Aiba S, Igarashi K, Okuyama R.** Bach1-dependent and -independent regulation of heme oxygenase-1 in keratinocytes. *J Biol Chem* 285: 23581–23589, 2010.
32. **Otterbein LE, Bach FH, Alam J, Soares M, Tao Lu H, Wysk M, Davis RJ, Flavell RA, Choi AM.** Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. *Nat Med* 6: 422–428, 2000.
33. **Oyake T, Itoh K, Motohashi H, Hayashi N, Hoshino H, Nishizawa M, Yamamoto M, Igarashi K.** Bach proteins belong to a novel family of BTB-basic leucine zipper transcription factors that interact with MafK and regulate transcription through the NF-E2 site. *Mol Cell Biol* 16: 6083–6095, 1996.
34. **Pileggi A, Molano RD, Berney T, Cattan P, Vizzardelli C, Oliver R, Fraker C, Ricordi C, Pastori RL, Bach FH, Inverardi L.** Heme oxygenase-1 induction in islet cells results in protection from apoptosis and improved in vivo function after transplantation. *Diabetes* 50: 1983–1991, 2001.
35. **Robertson RP.** Chronic oxidative stress as a central mechanism for glucose toxicity in pancreatic islet beta cells in diabetes. *J Biol Chem* 279: 42351–42354, 2004.
36. **Roskams T, Yang SQ, Koteish A, Durnez A, DeVos R, Huang X, Achten R, Verslype C, Diehl AM.** Oxidative stress and oval cell accumulation in mice and humans with alcoholic and nonalcoholic fatty liver disease. *Am J Pathol* 163: 1301–1311, 2003.
37. **Rudich A, Tirosh A, Potashnik R, Hemi R, Kanety H, Bashan N.** Prolonged oxidative stress impairs insulin-induced GLUT4 translocation in 3T3-L1 adipocytes. *Diabetes* 47: 1562–1569, 1998.
38. **Sun J, Brand M, Zenke Y, Tashiro S, Groudine M, Igarashi K.** Heme regulates the dynamic exchange of Bach1 and NF-E2-related factors in the Maf transcription factor network. *Proc Natl Acad Sci USA* 101: 1461–1466, 2004.
39. **Sun J, Hoshino H, Takaku K, Nakajima O, Muto A, Suzuki H, Tashiro S, Takahashi S, Shibahara S, Alam J, Taketo MM, Yamamoto M, Igarashi K.** Hemoprotein Bach1 regulates enhancer availability of heme oxygenase-1 gene. *EMBO J* 21: 5216–5224, 2002.
40. **Suttner DM, Dennery PA.** Reversal of HO-1 related cytoprotection with increased expression is due to reactive iron. *FASEB J* 13: 1800–1809, 1999.
41. **Suzuki T, Imai J, Yamada T, Ishigaki Y, Kaneko K, Uno K, Hasegawa Y, Ishihara H, Oka Y, Katagiri H.** Interleukin-6 enhances glucose-stimulated insulin secretion from pancreatic beta-cells: potential involvement of the PLC-IP3-dependent pathway. *Diabetes* 60: 537–547, 2011.
42. **Takahashi T, Shimizu H, Morimatsu H, Inoue K, Akagi R, Morita K, Sassa S.** Heme oxygenase-1: a fundamental guardian against oxidative tissue injuries in acute inflammation. *Mini Rev Med Chem* 7: 745–753, 2007.
43. **Tanimoto T, Hattori N, Senoo T, Furonaka M, Ishikawa N, Fujitaka K, Haruta Y, Yokoyama A, Igarashi K, Kohno N.** Genetic ablation of the Bach1 gene reduces hyperoxic lung injury in mice: role of IL-6. *Free Radic Biol Med* 46: 1119–1126, 2009.
44. **Vasir B, Aiello LP, Yoon KH, Quickel RR, Bonner-Weir S, Weir GC.** Hypoxia induces vascular endothelial growth factor gene and protein expression in cultured rat islet cells. *Diabetes* 47: 1894–1903, 1998.
45. **Watari Y, Yamamoto Y, Brydun A, Ishida T, Mito S, Yoshizumi M, Igarashi K, Chayama K, Ohshima T, Ozono R.** Ablation of the bach1 gene leads to the suppression of atherosclerosis in bach1 and apolipoprotein E double knockout mice. *Hypertens Res* 31: 783–792, 2008.
46. **Wellen KE, Hotamisligil GS.** Inflammation, stress, and diabetes. *J Clin Invest* 115: 1111–1119, 2005.
47. **Weyer C, Bogardus C, Mott DM, Pratley RE.** The natural history of insulin secretory dysfunction and insulin resistance in the pathogenesis of type 2 diabetes mellitus. *J Clin Invest* 104: 787–794, 1999.
48. **Won KC, Moon JS, Eun MJ, Yoon JS, Chun KA, Cho IH, Kim YW, Lee HW.** A protective role for heme oxygenase-1 in INS-1 cells and rat islets that are exposed to high glucose conditions. *J Korean Med Sci* 21: 418–424, 2006.
49. **Xiang FL, Lu X, Strutt B, Hill DJ, Feng Q.** NOX2 deficiency protects against streptozotocin-induced beta-cell destruction and development of diabetes in mice. *Diabetes* 59: 2603–2611, 2010.
50. **Yamaguchi S, Ishihara H, Yamada T, Tamura A, Usui M, Tominaga R, Munakata Y, Satake C, Katagiri H, Tashiro F, Aburatani H, Tsukiyama-Kohara K, Miyazaki J, Sonenberg N, Oka Y.** ATF4-mediated induction of 4E-BP1 contributes to pancreatic beta cell survival under endoplasmic reticulum stress. *Cell Metab* 7: 269–276, 2008.
51. **Yano Y, Ozono R, Oishi Y, Kambe M, Yoshizumi M, Ishida T, Omura S, Oshima T, Igarashi K.** Genetic ablation of the transcription repressor Bach1 leads to myocardial protection against ischemia/reperfusion in mice. *Genes Cells* 11: 791–803, 2006.