THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 31, pp. 23581–23589, July 30, 2010

Bach1-dependent and -independent Regulation of Heme Oxygenase-1 in Keratinocytes*

Received for publication, September 20, 2009, and in revised form, April 26, 2010 Published, JBC Papers in Press, May 25, 2010, DOI 10.1074/jbc.M109.068197

Shuko Okada[‡], Akihiko Muto[§], Eisaku Ogawa[‡], Ayako Nakanome[§], Yasutake Katoh[§], Shuntaro Ikawa[¶], Setsuya Aiba[‡], Kazuhiko Igarashi[§], and Ryuhei Okuyama[‡‖1]

From the Departments of [‡]Dermatology and [§]Biochemistry, Tohoku University Graduate School of Medicine, Sendai 980-8574, the [¶]Ikawa Group, Center for Interdisciplinary Research, Tohoku University, Sendai 980-8578, and the [‖]Department of Dermatology, Shinshu University School of Medicine, Matsumoto 390-8621, Japan

Bach1 is a member of the basic leucine zipper transcription factor family, and the Bach1/small Maf heterodimer specifically represses transcriptional activity directed by the Maf recognition element (MARE). Because Bach1 is a repressor of the oxidative stress response, we examined the function(s) of Bach1 in keratinocytes subjected to oxidative stress. Oxidative stress induced by H_2O_2 led to an increase in MARE activity and expression of heme oxygenase-1 (HO-1), an inducible antioxidant defense enzyme. Bach1 depletion by small interfering RNAs or by deletion of *Bach1* enhanced HO-1 expression in the absence of H_2O_2, indicating that Bach1 is a critical repressor of HO-1 in keratinocytes. Although Bach1-deficient or -reduced keratinocytes expressed higher levels of HO-1 than control cells in response to H_2O_2, Bach1 down-regulation did not attenuate the production of reactive oxygen species by H_2O_2. In contrast, Bach1 overexpression abolished HO-1 induction by H_2O_2, which led to increased reactive oxygen species accumulation. HO-1 was induced during keratinocyte differentiation, but MARE activity did not change during differentiation. Furthermore, Bach1 overexpression did not inhibit differentiation-associated induction of HO-1 expression, suggesting that HO-1 induction in differentiation is independent of Bach1. Thus, in response to oxidative stress, Bach1 regulates the oxidation state through the negative control of HO-1 expression prior to terminal keratinocyte differentiation. However, Bach1-mediated repression is negated during keratinocyte differentiation.

Redox regulation is critical to cell survival, because cells cannot avoid endogenous reactive oxygen species (ROS)[2] that are generated as by-products of respiration (1). The skin is constantly exposed to harmful ROS that are generated by pro-oxidant agents in the environment, as well as by endogenous cellular oxidants. Thus, keratinocytes strongly and constitutively express ROS-detoxifying enzymes, including superoxide dismutase, catalase, glutathione peroxidase, and reductase, and contain substantial levels of the antioxidants tocopherol and ubiquinol (2, 3). Additionally, these cells possess an inducible defense system that includes heme oxygenase-1 (HO-1) (3–5). An imbalance between ROS and antioxidants can lead to the generation of high oxidant levels, which play a pivotal role in skin aging and disease (6).

The transcription factor Bach1 is a repressor of the oxidative stress response in higher eukaryotes (7, 8) and, together with its paralogue Bach2, constitutes a subfamily of the basic leucine zipper family of proteins. Bach1 forms heterodimers with the basic leucine zipper subfamily of small Maf proteins (*i.e.* MafF, MafG, and MafK), which bind the Maf recognition element (MARE) in the promoter regions of genes such as *HO-1*, NADP(H) quinone (oxido)reductase, and β-globin (7, 9, 10), and thereby repress transcription in the absence of oxidative stress. In the presence of oxidative stress, Bach1 is inactivated (11, 12), which allows transcriptional activation of these genes by Nrf2 and other activators that also form heterodimers with small Maf proteins (7, 13). Thus, redox regulation is partly mediated by reciprocal DNA-MARE binding by the Bach1 repressor and other transcriptional activators.

Heme oxygenases HO-1 and HO-2 are the rate-limiting enzymes in heme degradation (14). HO-1 is an inducible enzyme, and HO-2 is constitutively expressed. Heme is a critical prosthetic group for many organisms and is synthesized in virtually all cells. However, free heme is a potent pro-oxidant that catalyzes the formation of ROS by the Fenton reaction (14). HO-1 converts excess heme into ferrous iron, carbon monoxide, and biliverdin, which is reduced to bilirubin by biliverdin reductase. Carbon monoxide and biliverdin, as well as bilirubin, have anti-oxidant and anti-inflammatory properties *in vivo* (15, 16), suggesting a role for HO-1 in the conversion of heme into antioxidants for defense against ROS. The transcription of *Hmox-1*, which encodes HO-1, is induced by oxidative stress (14). As the inducible enhancers of *Hmox-1* carry multiple MAREs, HO-1 transcription is regulated by the balance between Bach1 and transcriptional activators.

The epidermis constitutes the primary defense mechanism of the body against oxidative damage. HO-1 is induced during keratinocyte differentiation and is highly expressed in the upper layers of the epidermis (17). However, the mechanism of Bach1-mediated HO-1 regulation in keratinocytes

* This work was supported in part by a grant-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology of the Japanese Government.

[1] To whom correspondence should be addressed: 3-1-1 Asahi, Matsumoto, Nagano 390-8621, Japan. Fax: 81-263-37-2646; E-mail: rokuyama@shinshu-u.ac.jp.

[2] The abbreviations used are: ROS, reactive oxygen species; MARE, Maf recognition element; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; PKC, protein kinase C; siRNA, small interfering RNA; m.o.i., multiplicity of infection; ChIP, chromatin immunoprecipitation; RNAi, RNA interference; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate.

remains unclear. Here, we investigated the role of Bach1 in HO-1 regulation and ROS production.

EXPERIMENTAL PROCEDURES

Plasmids and Antibodies—The Bach1 expression plasmid (plasmid pCMV/Bach1) has been described previously (8). The pRBGP3 and pRBGP2 plasmids contain no copies or three copies, respectively, of the MARE region (5′-TCG ACC CGA AAG GAG CTG ACT CAT GCT AGC CC-3′) upstream of the thymidine kinase promoter in pGL2-TK (18). The reporter plasmids for the regulatory regions of *HO-1* (pHO15luc) and *Blimp1* possess MARE regions (10, 19, 20). The pHO15luc reporter plasmid contains the 15 kb of DNA upstream of the mouse *HO-1* gene and was previously shown to recapitulate inducible expression of the *HO-1* gene in response to heme, cadmium, and H_2O_2 (19). Polyclonal antiserum against Bach1 (A1–6) was produced as described previously (21). The following antibodies were purchased and used as recommended by their suppliers: rabbit anti-HO-1 (Abcam, Tokyo, Japan); goat anti-lamin B (Santa Cruz Biotechnology, Tokyo, Japan); and mouse monoclonal anti-α-tubulin (Sigma). Horseradish peroxidase-conjugated goat anti-mouse IgG, goat anti-rabbit IgG (Amersham Biosciences), and rabbit anti-goat IgG (Santa Cruz Biotechnology) were used as secondary antibodies.

Mice, Cell Culture, Transient Transfection, and Adenoviral Infection—Primary keratinocytes were prepared from newborn ICR mouse epidermis, as described previously (22). In brief, the epidermis was separated from the dermis with 0.25% trypsin (Invitrogen) overnight at 4 °C, plated in dishes precoated with type I collagen (Nitta Gelatin, Osaka, Japan), and cultured in minimum essential medium supplemented with 4% Chelex-treated fetal calf serum, epidermal growth factor (10 ng/ml; Invitrogen), and 0.05 mM $CaCl_2$. Under these conditions, keratinocytes were maintained in an immature state, and differentiation was induced by addition of $CaCl_2$ to a final concentration of 2 mM, as described elsewhere (23). Primary keratinocytes were also prepared from the newborn mouse epidermis of *Bach1*$^{-/-}$ mice and *Bach1*$^{+/+}$ mice using the procedure described above. The generation of *Bach1*$^{-/-}$ mice has been described previously (10). The study was carried out in accordance with international ethical guidelines for laboratory animals and was approved by the Institutional Animal Care and Use Committee. The following reagents were purchased from the indicated commercial sources: H_2O_2 (Wako, Osaka, Japan), doxycycline and the γ-secretase inhibitor *N*-[*N*-(3,5-difluorophenylacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester (Sigma), H_2DCFDA (Invitrogen), the JNK inhibitor II, protein kinase C (PKC) α/β inhibitor Gö6976, the PKCδ/θ inhibitor rottlerin (Merck), the ERK inhibitor PD98059, and p38 MAPK inhibitor SB203580 (Cell Signaling Technology, Tokyo, Japan). The ATP assay kit was performed according to the supplier's instructions (Bioassay Systems, Hayward, CA).

Transfections were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. To assess promoter activity, transfected keratinocytes were subjected to a Dual-Luciferase reporter assay (Promega, Tokyo, Japan) 72 h after transfection. Relative luciferase activities were normalized to *Renilla* luciferase activity. The small interfering RNAs (siRNAs) specific to Bach1 (B-1, B-2, and B-3) were purchased from Invitrogen, together with a stealth siRNA as a control. The HO-1-specific siRNA sequences were as follows: HO-1 siRNA1, 5′-AUC ACC AGC UUA AAG CCU UCU CUG G-3′; HO-1 siRNA2, 5′-CCA GAG AAG GCU UUA AGC UGG UGA U-3′. Recombinant adenoviruses expressing either *Bach1* (Ad-Bach1) or *lacZ* (Ad-LacZ) were generated as described previously and used at a multiplicity of infection (m.o.i.) of 25 (22, 24). Ad-LacZ was used as a control. Bach1 expression was induced by a regulatory virus (Ad-TO; Adeno-X Tet-On; Clontech) with the addition of doxycycline (100 ng/ml).

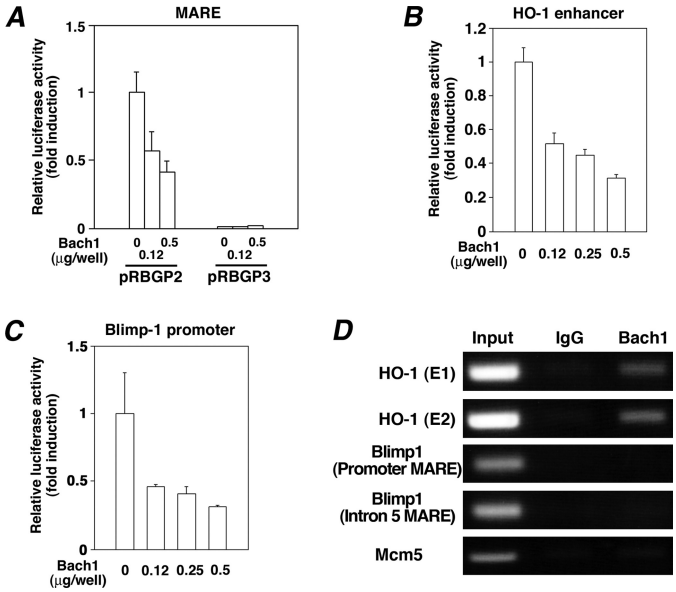

FIGURE 1. **Bach1 suppresses transactivation of MARE and gene promoters containing MARE regions.** *A,* Bach1 expression inhibits MARE activity in a dose-dependent manner. Primary keratinocytes were cultured in 12-well plates and cotransfected with increasing amounts of Bach1 expression plasmid and luciferase reporter plasmids, with or without MARE regions (pRBGP2 or pRBGP3). *B,* Bach1 expression inhibits the activity of the *HO-1* regulatory region in a dose-dependent manner. *C,* Bach1 expression inhibits the activity of the *Blimp1* regulatory region in a dose-dependent manner. *D,* Bach1 binds to the *HO-1* enhancers. Primary keratinocytes were subjected to ChIP analysis using antibodies against Bach1 and a nonspecific antibody as a control. The immunoprecipitated DNA was analyzed for enrichment of MARE motifs by PCR using primers amplifying DNA in the vicinity of the MARE motifs contained in the regulatory regions of the *HO-1* and *Blimp1* genes. The *Mcm5* promoter was used as a negative control. Similar results were obtained in at least two independent experiments.

Immunoblotting—Total cell proteins were extracted from cultured keratinocytes as described previously (22). Nuclear proteins were purified according to the manufacturer's protocol (nuclear extract kit; Active Motif, Tokyo, Japan). The extracted proteins were resolved by SDS-PAGE and transferred to an Immobilon-P membrane (Millipore, Tokyo, Japan). The membranes were blocked with TBST (50 mM Tris, pH 7.5, 0.5% Tween 20) containing 5% nonfat dried milk, incubated with primary antibodies, rinsed with TBST, and incubated with horseradish peroxidase-conjugated secondary antibodies. After additional rinses, the blots were exposed to LumiGLO reagent (Cell Signaling Technology) and subsequently to x-ray film. Protein concentrations were normalized using a BCA protein assay (Pierce), and equal loading was assessed by α-tubulin immunoblot.

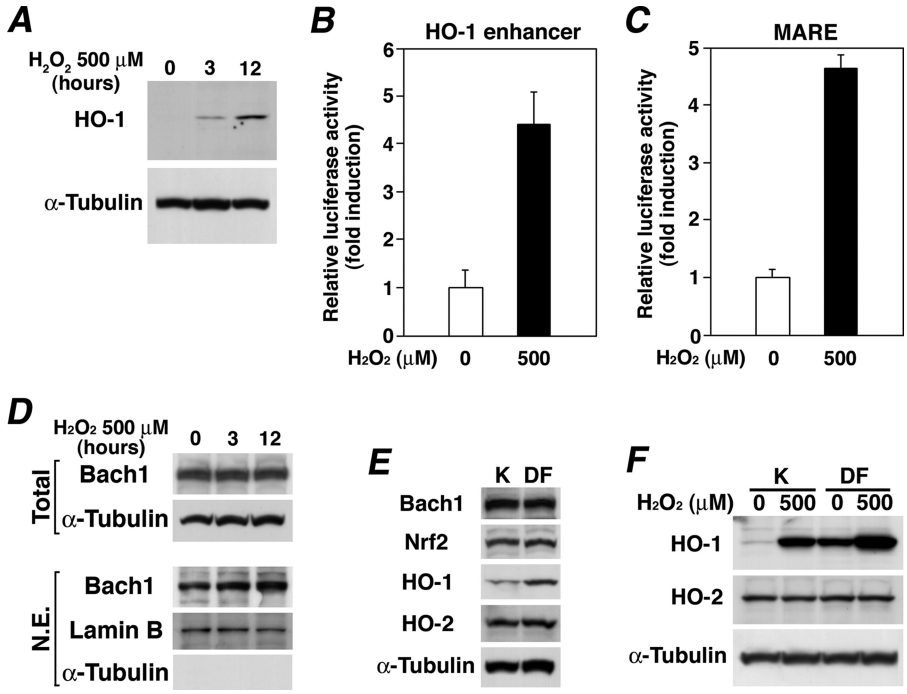

FIGURE 2. **H_2O_2 induces HO-1 expression due to increased MARE activity.** *A*, H_2O_2 induces HO-1 expression. Primary keratinocytes were incubated with 500 μM H_2O_2 for the indicated times. Protein levels were determined by immunoblotting with specific antibodies. The loading control was α-tubulin. *B*, H_2O_2 increases the activity of the *HO-1* regulatory region. Primary keratinocytes, transfected with a luciferase reporter plasmid containing the 15 kb of DNA upstream of the mouse *HO-1* gene (pHO15luc), were incubated in the presence or absence of 500 μM H_2O_2. *C*, H_2O_2 increases MARE activity. Primary keratinocytes transfected with the MARE containing a luciferase reporter plasmid (pRBGP2) were incubated in the presence or absence of 500 μM H_2O_2. *D*, Bach1 protein expression in response to H_2O_2. Primary keratinocytes were incubated with 500 μM H_2O_2 for the indicated times. Total and nuclear extracts (*N.E.*) were immunoblotted, and protein levels were determined by immunoblotting with specific antibodies. α-Tubulin and lamin B were used as loading controls in total and nuclear fractions, respectively. α-Tubulin in the nuclear fraction is a marker for the cytosolic fraction. *E*, basal expression of Bach1, Nrf2, HO-1, and HO-2 in keratinocytes (*K*) and dermal fibroblasts (*DF*). Total cell lysates obtained from primary keratinocytes and dermal fibroblasts were subjected to immunoblot analysis. The loading control was α-tubulin. *F*, H_2O_2-induced expression of HO-1 and HO-2. α-Tubulin was used as a loading control. Similar results were obtained in at least two independent experiments.

Real Time PCR—Total RNA was prepared from primary keratinocytes using the RNeasy mini kit (Qiagen, Tokyo) according to the manufacturer's instructions. The cDNAs were synthesized using SuperScript II reverse transcriptase (Invitrogen) and 3 μg of total RNA as a template. Real time PCR analyses were performed with a Quantitect SYBR Green PCR kit (Qiagen) and an iCycler (Bio-Rad) using the following primers: Bach1, 5′-GCC CGT ATG CTT GTG TGA TT-3′ and 5′-CGT GAG AGC GAA ATT ATC CG-3′; HO-1, 5′-GGG TGA CAG AAG AGG CTA AG-3′ and 5′-GTG TCT GGG ATG AGC TAG TG-3′; and Nrf2, 5′-CAA GAC TTG GGC CAC TTA AAA GAC-3′ and 5′-AGT AAG GCT TTC CAT CCT CAT CAC-3′.

Chromatin Immunoprecipitation (ChIP)—ChIP analyses were performed as described previously (25). In brief, keratinocytes were cross-linked with formaldehyde (1% final concentration) for 15 min at room temperature. Nuclear fractions were sonicated to break the DNA into fragments with an average length of ~200–500 bp and extracts subjected to immunoprecipitation with anti-Bach1 (A1–6) or normal rabbit serum. After releasing cross-linked DNA from the immunoprecipitated materials with 10 mM dithiothreitol, samples were incubated for 6 h at 65 °C to remove formaldehyde from the DNA. After purification with a QIAquick PCR purification kit (Qiagen), the recovered DNAs were subjected to PCR analyses. PCR analyses were performed using Takara LA Taq (Takara, Ohtsu, Japan) and an iCycler with primers capable of amplifying DNA fragments in the vicinity of the MARE motifs in the regulatory regions of *HO-1*, *Blimp1*, and *Mcm5*. The promoter of *Mcm5*, a gene adjacent to HO-1, was used as a control. The following primers were used for PCR amplification of reporters: HO-1 E1, 5′-TGA AGT TAA AGC CGT TCC GG-3′ and 5′-AGC GGC TGG AAT GCT GAG T-3′; HO-1 E2, 5′-GGG CTA GCA TGC GAA GTG AG-3′ and 5′-AGA CTC CGC CCT AAG GGT TC-3′; Blimp1 promoter MARE, 5′-CTG ACA CTT ACA TGG TAT CTG TGC CC-3′ and 5′-AAA TCC AGC CTC CTG CAG AGG-3′; and Blimp1 intron 5 MARE, 5′-GTT AAT CTG CTT TCT CGG TTT C-3′ and 5′-TCT TAA ATG GCT GTA GGC GGA C-3′. The *Mcm5* promoter primers were as described previously (25).

H_2DCFDA Staining—To quantify ROS, cultured keratinocytes were incubated with 100 or 500 μM H_2O_2 for 12 h and then stained with fluorescein isothiocyanate-conjugated H_2DCFDA. Analyses were performed using a FACSCalibur instrument with CellQuest software (BD Biosciences).

Statistical Analyses—Data were analyzed using an unpaired Student's *t* test. Differences were considered statistically significant for *p* values less than 0.05.

RESULTS

Inhibitory Effect of Bach1 on MARE Activity in Keratinocytes—First, we examined the effect of Bach1 on transactivation in keratinocytes. The activity of Bach1 in keratinocytes was accessed by transfecting them with the MARE reporter pRBG2 and a Bach1 expression plasmid. Exogenous Bach1 suppressed MARE activity in a dose-dependent manner (Fig. 1*A*), suggesting that Bach1 acts to repress gene function. Bach1 did not affect the basal activity of the promoter (pRBG3 lacking MARE). Furthermore, we analyzed the effects of Bach1 on transactivation of *HO-1* and *Blimp1*. Blimp1 is important for keratinocyte differentiation (26) and is repressed by Bach2, a paralogue of Bach1, in B lymphoid cells (27). The *HO-1* reporter pHO15luc contains a region of 15 kb of DNA upstream of the murine *HO-1* gene. The induction of HO-1 in response to heme and other stresses is regulated principally by two upstream enhancers within this 15-kb region, E1 and E2, both of which contain MAREs (19). The *Blimp1* promoter also possesses a

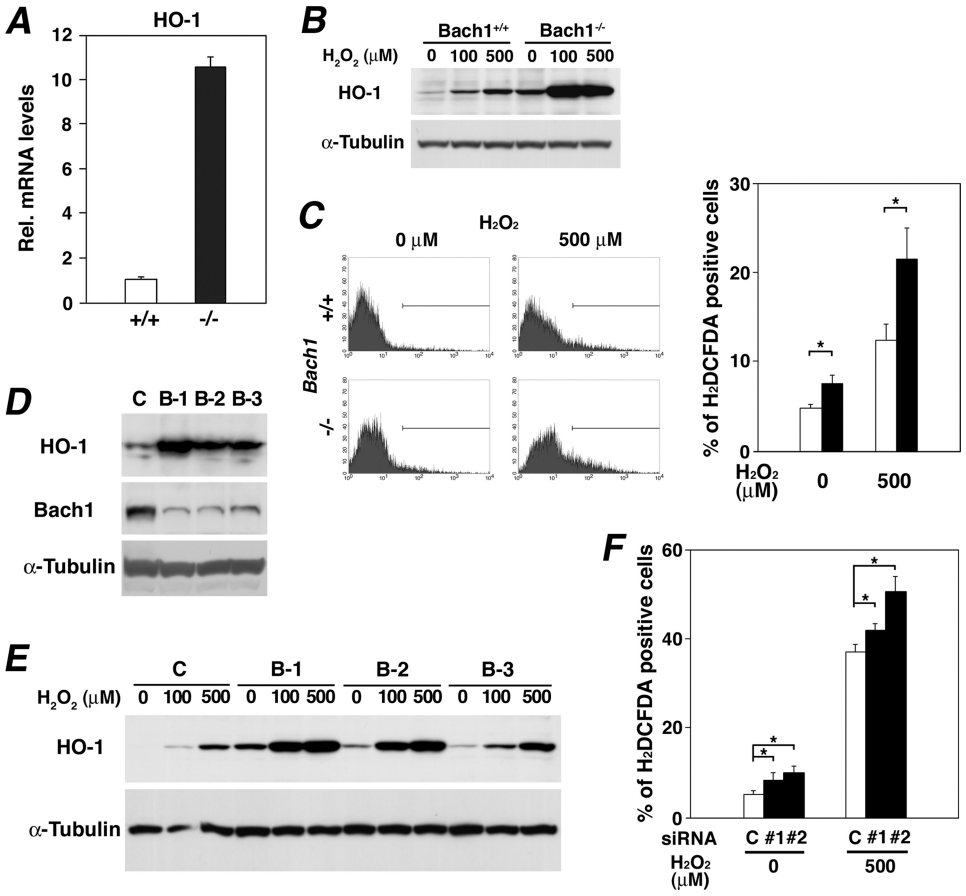

FIGURE 3. **Bach1 down-regulation does not decrease ROS levels despite the increase in HO-1 levels.** *A,* HO-1 mRNA levels of *Bach1*$^{-/-}$ (*solid bar*) *versus Bach1*$^{+/+}$ (*white bar*) keratinocytes. HO-1 mRNA levels were determined by real time reverse transcription-PCR with primers specific for the *HO-1* sequence. Values were normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA levels and are expressed as arbitrary units. *B,* HO-1 protein expression in *Bach1*$^{-/-}$ *versus Bach1*$^{+/+}$ keratinocytes. Keratinocytes were cultivated in the indicated concentrations of H_2O_2, and HO-1 expression was assessed by immunoblotting, with α-tubulin as a loading control. *C,* H_2DCFDA expression in *Bach1*$^{-/-}$ (*solid bar*) *versus Bach1*$^{+/+}$ (*white bar*) keratinocytes. Keratinocytes were incubated with H_2O_2 (0 or 500 μM) for 12 h. After H_2DCFDA addition, trypsinized keratinocytes were analyzed using a cell sorter (*left panel*). The ratio of ROS-positive cells to total cells is also shown (*right panel*). *Error bars* represent the standard deviations. *, $p < 0.05$. *D,* immunoblots of keratinocytes transfected with Bach1-specific siRNAs. HO-1 levels increased with Bach1 down-regulation by Bach1 sequence-specific (*B-1*, *B-2,* and *B-3*), but not control (*C*), siRNAs. The loading control was α-tubulin. *E,* H_2O_2 induces HO-1 expression in an additive manner in the siRNA-transfected keratinocytes. Primary keratinocytes transfected with specific siRNAs were incubated with the indicated concentrations of H_2O_2. HO-1 protein levels were determined by immunoblotting with anti-HO-1, and α-tubulin was used as a loading control. *F,* H_2DCFDA expression in keratinocytes transfected with Bach1 sequence-specific (*solid bar*; *B-1* and *B-2*) *versus* control siRNAs (*white bar*; *C*). Experiments were conducted as in *C*. Percentages indicate the ROS-positive fraction of the population. Standard deviations are indicated by *error bars*. *, $p < 0.05$. Similar results were obtained in at least two independent experiments.

MARE region (25). Bach1 repressed expression of both reporter genes (Fig. 1, *B* and *C*). Subsequently, we tested for binding of Bach1 to the *HO-1* enhancer regions (E1 and E2) and *Blimp1* regulatory regions (promoter MARE region and intron 5 MARE region (28)) by ChIP analysis (Fig. 1*D*). Bach1 bound to the E1 and E2 *HO-1* enhancer regions. In contrast, no association of Bach1 with the *Blimp1* regulatory regions was detected. However, we could not exclude the possibility of an association between Bach1 and the *Blimp1* regulatory region. The association of MafK with the *Blimp1* regulatory region was much weaker than its association with the *HO-1* enhancer, as detected by ChIP assay (data not shown). Therefore, the affinity of Bach1 for the *Blimp1* regulatory region may be lower than that for the *HO-1* enhancer.

Bach1 Regulation of HO-1 Expression in Response to Oxidative Stress—Because we detected a direct association between Bach1 and the *HO-1* enhancer region, we next focused on oxidative stress-induced expression of HO-1. H_2O_2 increased both the amount of HO-1 protein (Fig. 2*A*) and the activity of the *HO-1* enhancer region (Fig. 2*B*). MARE activation also increased in response to H_2O_2 (Fig. 2*C*). The increased MARE activity was consistent with HO-1 induction, because the *HO-1* enhancer contains multiple MAREs. Bach1 activity is regulated at various levels, including ubiquitin-dependent degradation, nuclear localization, and changes in DNA affinity (7, 29). Next, we examined the amount of Bach1 in total and nuclear cellular fractions. Bach1 levels remained constant in total cellular fraction despite H_2O_2 addition and were slightly increased in nuclear cellular fraction (Fig. 2*D*), suggesting that neither degradation nor nuclear export contributed to the increase in MARE activation. Because Bach1 is regulated by the redox status of its cysteine residues, this regulation is likely in response to H_2O_2 (11).

In addition, we compared the levels of Bach1, Nrf2, HO-1, and HO-2 expression in primary keratinocytes and in primary dermal fibroblasts. Bach1 is expressed ubiquitously (8) and in keratinocytes (30, 31). Immunoblot analysis revealed that Bach1 levels were similar in primary keratinocytes and primary dermal fibroblasts (Fig. 2*E*). Nrf2 was also expressed at similar levels in both cell types. However, keratinocytes exhibited lower levels of HO-1 compared with dermal fibroblasts. Furthermore, lower HO-1 levels were present after treatment with H_2O_2 in keratinocytes (Fig. 2*F*). A similar level of HO-2 expression in these cells suggests that the differential expression of HO-1 is not a result of alterations in HO-2 expression. Although the levels of Bach1 and Nrf2 in keratinocytes were similar to those in dermal fibroblasts, Bach1 appeared to be the defining feature for the regulation of HO-1 expression in keratinocytes.

Bach1 Down-regulation Enhances HO-1 Expression but Does Not Reduce or Attenuate ROS Production—To investigate the function of Bach1 in the regulation of HO-1 in keratinocytes, we examined the levels of ROS in *Bach1*$^{-/-}$ keratinocytes. Consistent with earlier results showing that Bach1

negatively regulates expression of the HO-1 reporter gene (Fig. 1*B*), *Bach1*$^{-/-}$ keratinocytes expressed higher levels of HO-1 mRNA and protein than wild-type cells under normal conditions (Fig. 3, *A* and *B*). Furthermore, H_2O_2 treatment resulted in much higher levels of HO-1 in *Bach1*$^{-/-}$ keratinocytes (Fig. 3*B*) than HO-1 levels in wild-type keratinocytes. These results indicate that Bach1 was responsible not only for the repression of basal HO-1 levels but also for the attenuation of HO-1 expression induced by oxidative stress conditions. Because increased HO-1 levels are predicted to protect against ROS, we examined whether *Bach1* deletion and subsequent HO-1 increase was sufficient to protect keratinocytes against oxidative stress. ROS levels increase within the cell upon treatment with H_2O_2. However, despite the higher level of HO-1 expression, ROS accumulation was not attenuated by the loss of Bach1 (Fig. 3*C*). Rather, ROS levels were higher in *Bach1*$^{-/-}$ keratinocytes than control cells, regardless of H_2O_2 treatment.

To check effects of Bach1 down-regulation on ROS levels further, we next compared HO-1 expression before and after Bach1 knockdown by RNA interference (RNAi). Bach1 was knocked down by transient transfection with siRNA constructs that targeted three different regions of the Bach1 transcript. Similar to the *Bach1*$^{-/-}$ keratinocytes, reduced Bach1 levels resulted in increased HO-1 expression (Fig. 3*D*). H_2O_2 treatment resulted in higher levels of HO-1 in Bach1-depleted keratinocytes (Fig. 3*E*), but ROS accumulation was not decreased by Bach1 depletion (Fig. 3*F*). Similar to *Bach1* gene deletion, RNAi inhibition of Bach1 expression increased ROS levels in the presence or absence of H_2O_2 treatment. Taken together, these results suggest that Bach1 down-regulation enhances HO-1 expression but is not sufficient to suppress ROS production.

To analyze the downstream effects of Bach1 down-regulated keratinocytes, we examined cell viability. Regardless of oxidative stress, the Bach1 down-regulation resulted in lowered keratinocyte viability (Fig. 4*A*). Furthermore, we quantified ATP levels because several heme proteins function in the mitochondria for energy production. The reduced Bach1 levels caused a decrease of ATP levels in both normal and oxidative stress conditions (Fig. 4*B*). These results suggest that HO-1 overexpression may lead to a disruption of energy production rather than protection against oxidative stress.

We further examined the effects of combined knockdown of Bach1 and HO-1 in keratinocytes by using HO-1-specific siRNA constructs. RNAi-mediated suppression of HO-1 mRNA decreased HO-1 levels even in the Bach1-depleted keratinocytes, regardless of oxidative stress (Fig. 4, *C* and *D*). The HO-1 knockdown attenuated the decreased cell viability by the Bach1 knockdown in both normal and oxidative stress conditions (Fig. 4*E*). In addition, the reduced HO-1 levels also abolished the decreased ATP levels in the Bach1-depleted keratinocytes (Fig. 4*F*). Thus, Bach1 appears to suppress HO-1 induction to maintain cellular homeostasis, particularly in energy production.

Bach1 Up-regulation Enhances ROS Production by Blocking HO-1-induced Expression—We next used an adenoviral transduction system to examine the effects of Bach1 overexpression. Using the Tet-On system, Bach1 expression was efficiently

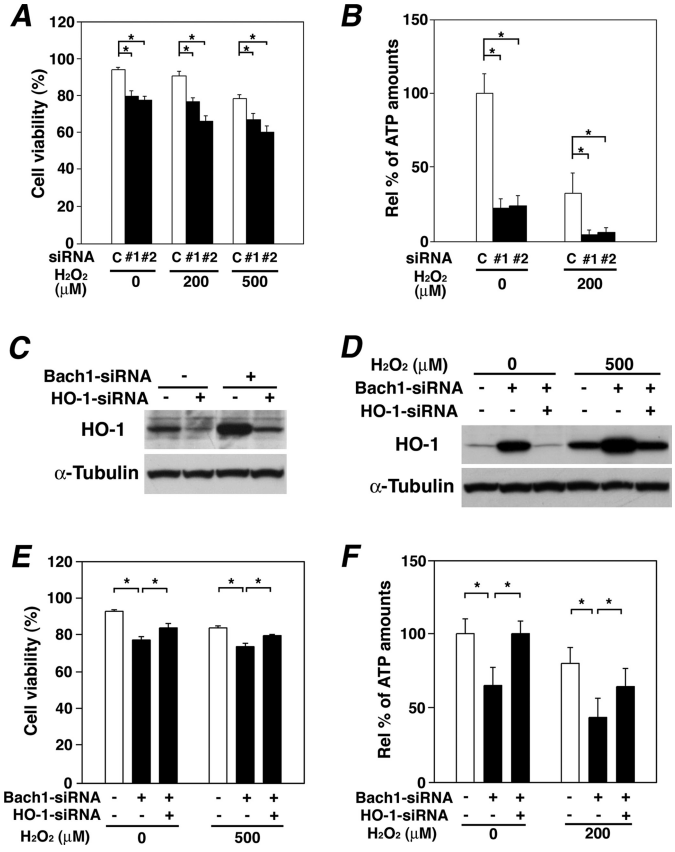

FIGURE 4. **Bach1 down-regulation reduces cell viability due to the increase in HO-1 levels.** *A,* cell viability in Bach1-knockdown keratinocytes (*solid bar*; *#1* and *#2*) *versus* control cells (*white bar*; *C*). Keratinocytes were transfected with Bach1-specific siRNA (0.6 μg/well) and were incubated with H_2O_2 (0, 200, or 500 μM) for 12 h. Trypan blue staining was performed. The number of trypan blue-excluded cells was calculated by counting a minimum of six fields. Standard deviations are shown by the *error bars*. *, $p < 0.05$. *B,* ATP levels in Bach1 knockdown keratinocytes (*solid bar*; *#1* and *#2*) *versus* control cells (*white bar*; *C*). Keratinocytes were cultivated on 96-well plates and were transfected with Bach1-specific or control siRNA (75 ng/well). Cells were incubated with H_2O_2 (0 or 200 μM) for 12 h. Cell lysates were reacted with D-luciferin for ATP quantification assays. Five or more wells were assayed per sample. Values are expressed as percentages relative to ATP level in cells transfected with control siRNA. Standard deviations are shown by the *error bars*. *, $p < 0.05$. *C,* immunoblots of keratinocytes transfected with Bach1- and/or HO-1-specific siRNAs. HO-1 levels were decreased by HO-1 sequence-specific, but not control, siRNA. The loading control was α-tubulin. *D,* HO-1-specific siRNA down-regulates HO-1 expression even in H_2O_2 addition. Primary keratinocytes transfected with specific siRNAs were incubated with the indicated concentration of H_2O_2. HO-1 protein levels were determined by immunoblotting with anti-HO-1, and α-tubulin was used as a loading control. *E,* cell viability in Bach1- and/or HO-1-knockdown keratinocytes *versus* control cells. Keratinocytes were transfected with Bach1- and/or HO-1-specific siRNAs (every 0.3 μg/well), and total siRNA amounts were compensated by control siRNA. The number of trypan blue-excluded cells was calculated as in *A*. *, $p < 0.05$. *F,* ATP levels in Bach1- and/or HO-1-knockdown keratinocytes *versus* control cells. Keratinocytes were transfected with Bach1- and/or HO-1-specific siRNAs (every 37 ng/well), and total siRNA amounts were compensated by control siRNA. ATP quantification assay was performed as in *B*. *, $p < 0.05$. Similar results were obtained in at least two independent experiments.

induced in keratinocytes (Fig. 5*A*). In the absence of oxidative stress, Bach1 overexpression did not alter the basal level of HO-1 expression in keratinocytes (Fig. 5*B*), showing that endogenous HO-1 expression is not dependent on the MARE elements in the E1 and E2 enhancer regions. In contrast, HO-1 induction in response to H_2O_2 was almost completely inhibited by overexpression of Bach1 in keratinocytes (Fig. 5*C*). Consequently, ROS levels were much higher in Bach1-overexpressing

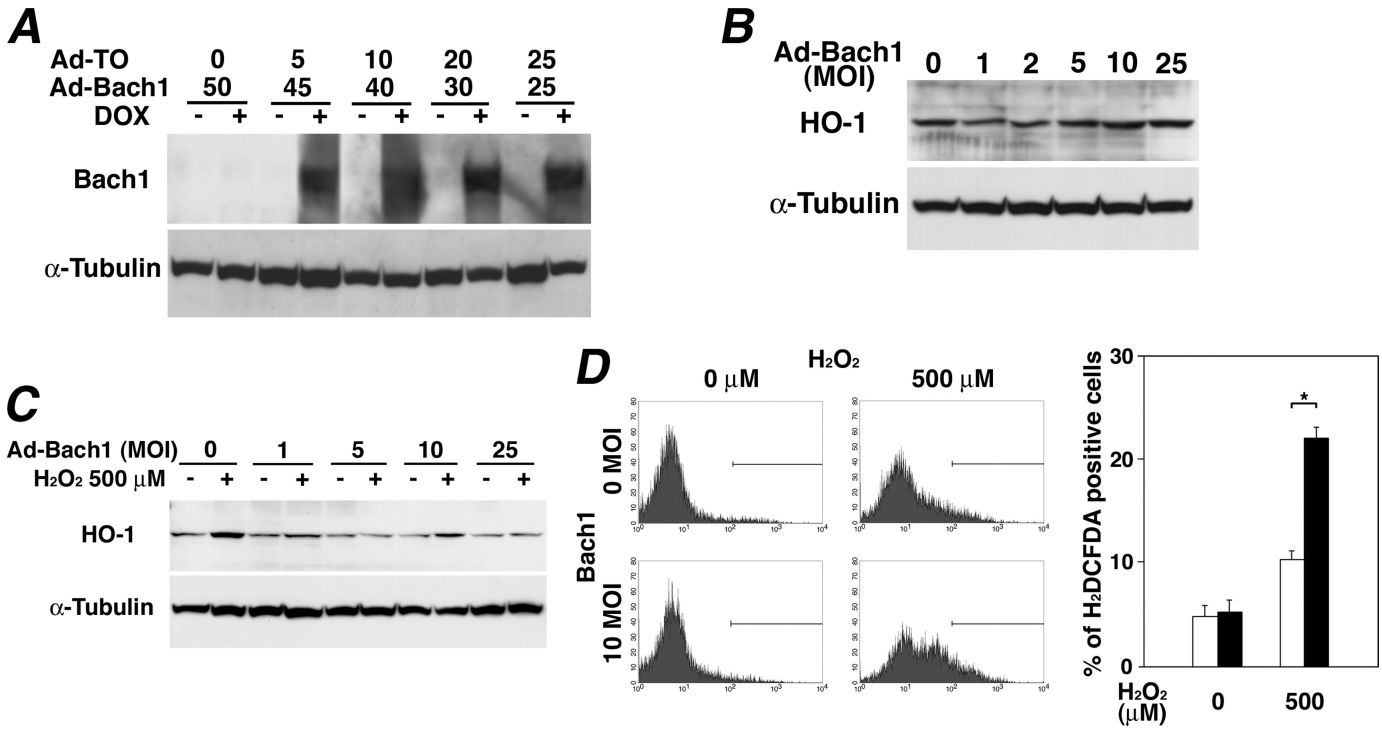

FIGURE 5. **Bach1 up-regulation leads to increased ROS levels due to insufficient HO-1 induction.** *A,* Bach1 overexpression in keratinocytes. Keratinocytes were infected with the indicated m.o.i. of adenoviruses expressing Bach1 (*Ad-Bach1*) and the reverse tetracycline-controlled repressor (*Ad-TO*), which turns on Bach1 transcription in the presence of doxycycline (*DOX*). Cell extracts were immunoblotted with anti-Bach1. The loading control was α-tubulin. *B,* Bach1 overexpression does not alter HO-1 expression. Keratinocytes coinfected with increasing m.o.i. (*MOI*) of Ad-Bach1 and a 25 m.o.i. of Ad-TO were compensated for a total of 50 m.o.i. by infection with an adenovirus expressing LacZ. Cells were cultivated in DOX-containing medium. The loading control was α-tubulin. *C,* Bach1 overexpression prevents H_2O_2-induced HO-1 expression. Experiments were conducted as in *B*. Keratinocytes were incubated in the presence or absence of 500 μM H_2O_2. The loading control was α-tubulin. *D,* H_2DCFDA expression in Bach1-overexpressing (*solid bar*) *versus* control keratinocytes (*white bar*). Keratinocytes were infected with Ad-Bach1 and Ad-TO and were followed by the addition of DOX, as in *B*. After incubation in the presence or absence of 500 μM H_2O_2 for 12 h, trypsinized keratinocytes were analyzed using a cell sorter (*left panel*). The ratio of ROS-positive cells to the number of total cells is also shown (*right panel*). Standard deviations are indicated by *error bars*. *, $p < 0.05$. The results shown are representative of at least two independent experiments.

cells than in control cells (Fig. 5*D*). Taken together, these results suggest that Bach1-mediated repression of HO-1 is specific to stress-induced HO-1 expression and was not effective in regulation of basal HO-1 levels under normal culture conditions. Interestingly, Bach1 up-regulation abolished cellular responses against oxidative stress, which resulted in ROS accumulation.

Bach1-HO-1 Pathway during Keratinocyte Differentiation—We next examined the role of Bach1 in the regulation of HO-1 during keratinocyte differentiation. Upon transfer of keratinocytes to high calcium medium, thereby forcing differentiation and the expression of keratins 1 and 10, loricrin, and filaggrin, the expression of HO-1 gradually and steadily increased at both the mRNA and protein levels (Fig. 6, *A* and *B*). In contrast, MARE reporter activity and HO-1 reporter activity did not increase during this differentiation period (Fig. 6, *C* and *D*). In addition, the level of the Bach1 transcript remained constant over this process (Fig. 6*E*). Similar to Bach1 levels, Nrf2 mRNA levels remained constant during differentiation. The protein levels of Bach1 and Nrf2 were increased in the nuclear fraction (Fig. 6*F*). Thus, HO-1 expression was induced by keratinocyte differentiation, but this induction was independent of both increased MARE activity and derepression of Bach1-mediated inhibition of HO-1 expression.

To further confirm that the differentiation-induced HO-1 expression was independent of Bach1, we overexpressed Bach1 in keratinocytes and examined HO-1 expression. The overexpression of Bach1 did not inhibit the calcium-induced expression of HO-1 (Fig. 6*G*). Furthermore, Bach1 did not affect basal HO-1 expression (*i.e.* in low calcium medium), supporting the finding that Bach1 is not relevant to HO-1 regulation under normal conditions. Thus, HO-1 induction is independent of Bach regulation during keratinocyte differentiation.

We next examined the impact of several signaling pathways on HO-1 induction during differentiation by the use of specific inhibitors. HO-1 induction was enhanced by inhibition of JNK, p38 MAPK, PKCδ/θ, or γ-secretase that is necessary for Notch activation (Fig. 7). These signal pathways appeared to suppress HO-1 induction during differentiation. On the other hand, neither ERK nor PKCα/β inhibitors affected HO-1 induction. Thus, HO-1 expression seemed to be repressed by several pathways that are known to regulate keratinocyte differentiation. The putative signaling pathway for accelerating HO-1 induction during calcium-induced differentiation remains to be identified.

DISCUSSION

Keratinocytes are constantly exposed to environmental prooxidants, which lead to harmful effects in the absence of appropriate protective cellular responses. Bach1 acts as a redox-sensitive repressor of genes that are under the control of the MARE element, but the role Bach1 plays in the environmental stress response in keratinocytes has not yet been reported. In this

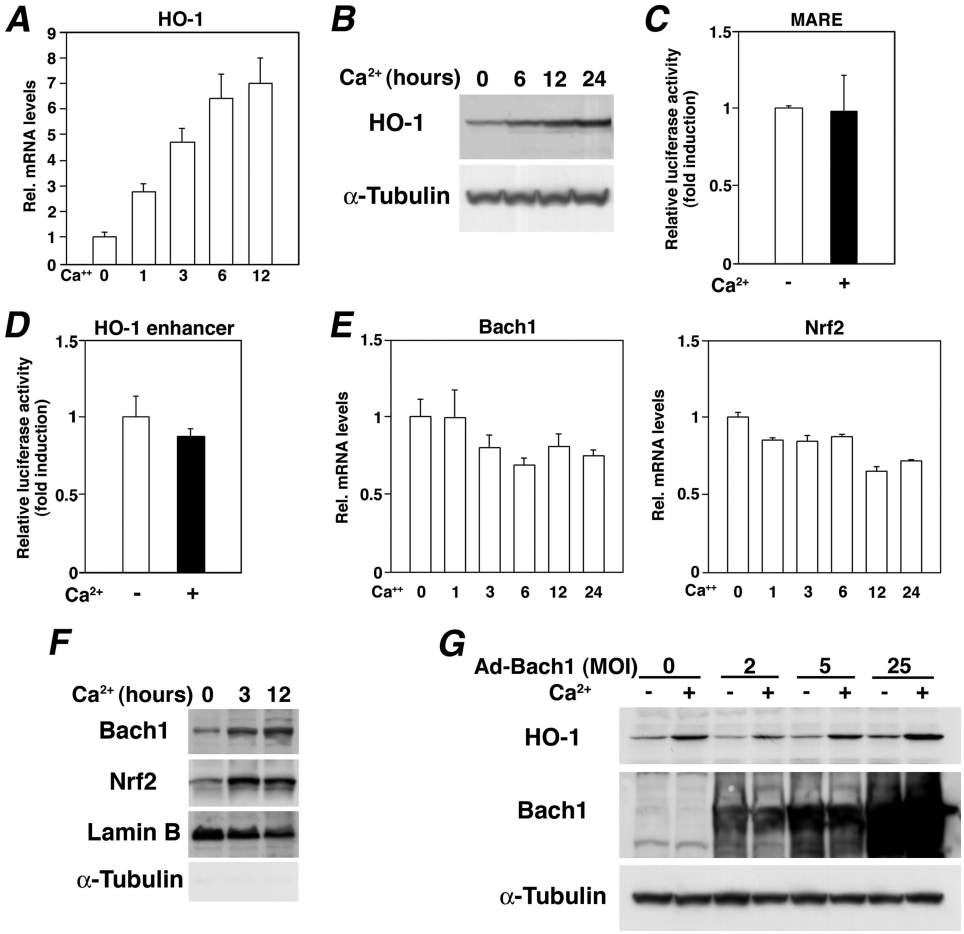

FIGURE 6. **HO-1 is induced independently of Bach1 inactivation during keratinocyte differentiation.** *A,* HO-1 transcription increases gradually in differentiating keratinocytes. RNA extracts were prepared from keratinocytes cultivated for the indicated hours after transfer to high calcium medium (2 mM). HO-1 mRNA levels were determined by real time reverse transcription-PCR. Values were normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA levels and are expressed as arbitrary units. *B,* HO-1 protein gradually increases in differentiating keratinocytes. Keratinocyte cultures were as in *A*. Total cell extracts were collected, and the proteins were immunoblotted with antibodies against HO-1 and α-tubulin as a loading control. *C,* MARE activity is stable independently of differentiation state. Primary keratinocytes transfected with a luciferase reporter plasmid carrying the wild-type MARE (pBRGP2) were incubated in the presence or absence of high calcium (2 mM) medium. *D,* transactivation of the *HO-1* gene is stable, irrespective of differentiation state. Keratinocytes were transfected with a reporter plasmid carrying the 15 kb of DNA upstream of the *HO-1* regulatory region. *E,* Bach1 and Nrf2 mRNA expression levels during keratinocyte differentiation. Primary keratinocytes were cultivated and mRNA levels were determined as in *A*. *F,* protein expression of Bach1 and Nrf2 in the nuclear fraction increase during keratinocyte differentiation. The nuclear fraction was immunoblotted with antibodies against Bach1 and Nrf2. Blots were stripped and reprobed with antibodies against lamin B and α-tubulin. Lamin B is a control for equal loading, and α-tubulin is a marker for cytosol fraction. *G,* calcium induces HO-1 expression irrespective of Bach1 overexpression. Keratinocytes coinfected with an increasing m.o.i. of Ad-Bach1 and 25 m.o.i. of Ad-TO were compensated for a total of 50 m.o.i. by an adenovirus expressing LacZ. Cells were cultivated in DOX-containing medium. Keratinocytes were incubated in the presence or absence of high calcium. The loading control was α-tubulin. Similar results were obtained in at least two independent experiments.

study, we used keratinocytes with depleted Bach1 levels to demonstrate that Bach1 inhibits the oxidative stress-inducible expression of HO-1, a cytoprotective enzyme.

Although deletion of the *Bach1* gene or RNAi resulted in higher expression of HO-1, Bach1 overexpression did not affect basal HO-1 expression. These apparently conflicting results are reconciled by the fact that even under normal culture conditions, cells are exposed to oxidative stress due to the presence of ~20% oxygen in the atmosphere (32). We hypothesize that the levels of Bach1 were sufficient to inhibit HO-1 induction in response to oxidative stress.

Because Bach1 and its competitors such as Nrf2 share small Maf proteins as their obligate heterodimer partners, a reduction in Bach1 levels would shift the equilibrium toward the formation of Nrf2/small Maf heterodimers, resulting in constitutively high levels of HO-1 expression. Hence, HO-1 may be readily activated by Nrf2 or other MARE-binding activators in Bach1-depleted keratinocytes. The higher levels of HO-1 generated by Bach1 down-regulation would protect cells against excessive oxidative damage. However, despite enhanced HO-1 expression, ROS levels in cultured keratinocytes were not decreased in *Bach1*-deficient keratinocytes or following RNAi depletion of Bach1. Rather, RNAi-mediated Bach1 down-regulation was sufficient to increase ROS levels. Therefore, there may be a limit to the cytoprotective function of HO-1 in keratinocytes. Because the cytoprotective function of HO-1 is manifested by reaction products such as bilirubin and carbon monoxide (14), the heme substrate could be the limiting factor in HO-1 function. Cells usually contain very little free heme (33), because the free heme molecule is cytotoxic due to its ability to catalyze the peroxidation of membrane lipids and formation of ROS. Higher HO-1 may promote degradation of not only free heme but also heme contained in heme protein complexes, leading to dysfunction of heme proteins. The increase of HO-1 levels by RNAi depletion of Bach1 resulted in a decrease of cell viability and a decrease in ATP levels in the presence or absence of oxidative stress, suggesting that HO-1 overexpression may lead to interference of energy production by degradation of mitochondrial heme proteins. The repressive effect of Bach1 appears to be of functional significance in the control of heme metabolism.

In contrast to keratinocytes, the higher levels of HO-1 found in Bach1-deficient cells may help protect and repair damaged tissues under particular conditions. Higher expression of HO-1 in *Bach1*$^{-/-}$ mice has been shown to lead to decreased tissue damage in diseases such as arteriosclerosis (34), ischemic reperfusion of the heart (35), and in spinal cord injury (36). In these models, heme may be released from damaged cells and used as a substrate for HO-1. Compared with dermal fibroblasts, kera-

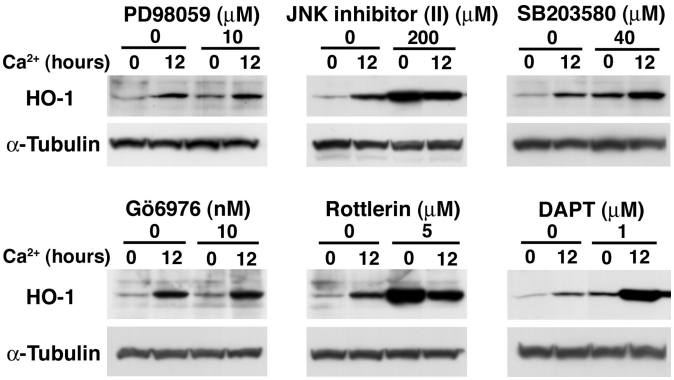

FIGURE 7. **HO-1 is controlled by several signal pathways during keratinocyte differentiation.** HO-1 induction is enhanced upon inhibition of JNK, p38 MAPK, PKCδ/θ, or Notch signaling. Keratinocytes were incubated with or without the ERK inhibitor PD98059, the JNK inhibitor II, the p38-MAPK inhibitor SB203580, the PKCα/β inhibitor Gö6976, the PKCδ/θ inhibitor rottlerin, or the γ-secretase inhibitor *N*-[*N*-(3,5-difluorophenylacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester (*DAPT*). Differentiation was induced by a shift from low to high calcium medium. α-Tubulin was used as a loading control. Similar results were obtained in at least two independent experiments.

tinocytes displayed a lower level of basal HO-1 expression with no changes in HO-2 levels, suggesting lower levels of total HO activity. Even under oxidative stress, HO-1 expression was lower in keratinocytes. The lower HO-1 may reflect a smaller pool of free heme in keratinocytes. Zhong *et al.* (31) has also reported a lower level of HO-1 in keratinocytes compared with dermal fibroblasts. Although the protein expression of Bach1 and Nrf2 did not differ between keratinocytes and dermal fibroblasts, Bach1 transcriptional activity may be dominant over Nrf2 activity in keratinocytes, which is compatible with previous reports that Bach1 inactivation is necessary and sufficient for HO-1 induction in the human HaCaT keratinocyte cell line (30).

The overexpression of Bach1 attenuated HO-1 induction under conditions of oxidative stress, which led to ROS accumulation. Indeed, *HO-1* null cells have been reported to be more sensitive to oxidative stress (37–41), and Bach1-overexpressing cells share certain features with *Nrf2*-deficient cells, which show high levels of cell death upon oxidative stress (42–44), suggesting that Bach1 overexpression predisposes cells to oxidative stress. Bach1 activity is negatively controlled through multiple mechanisms, including inhibition of DNA binding, induction of nuclear export, and degradation through the polyubiquitination pathway (7, 29). The stringent negative regulation of Bach1 through these mechanisms may be important for keeping Bach1 activity within a narrow range, because high levels of Bach1 promote cell death. Conversely, Bach1 inactivation in response to stress would induce target gene expression through the establishment of a higher activator-to-repressor ratio. The repression of HO-1 expression by Bach1 could result in a rapid and robust regulation of target transcription in response to changes in the environment.

In contrast to oxidative stress-induced regulation of HO-1, the differentiation-associated regulation of HO-1 in keratinocytes was found to be independent of Bach1 activity. The epidermis is organized into several distinct overlying layers. Actively proliferating keratinocytes are limited to the bottom, or basal, layer. As keratinocytes begin to differentiate, they cease cell growth and migrate to the upper layers (the spinous, granular, and cornified layers). HO-1 is induced during the terminal differentiation and is expressed in the upper part of the epidermis (17). These layers of the skin are directly exposed to the environment and require defense mechanisms against environmental insults, including oxidative stress and ultraviolet light. However, HO-1 induction during differentiation *in vitro* was independent of Bach1 inactivation and Nrf2 activation. Upon differentiation, HO-1 induction was not inhibited by Bach1 overexpression, suggesting that the differentiation-induced regulation of HO-1 is mediated by a MARE-independent mechanism under these conditions. *Keap1*-null mice, in which Nrf2 is constitutively activated, have a noticeably thicker cornified layer and express differentiation-specific proteins more abundantly than their wild-type littermates (45). Thus, Nrf2 overactivation alters some aspects of the differentiation process. However, it is unlikely that Nrf2 directly regulates keratinocyte differentiation under normal conditions, because the reporter activity of MARE was stable during normal differentiation. Instead, based on the observation that Nrf2 was activated in response to various stresses, the altered differentiation may be the result of an enhanced response to external stress, which would lead to increased keratinization as a defense. However, the mechanism responsible for the increased HO-1 expression in keratinocyte differentiation remains to be determined. Keratinocytes undergo complex changes during differentiation, which require the precise coordination of various molecular events (46). We did not identify a signaling pathway that accelerated HO-1 expression in differentiating keratinocytes. Unexpectedly, we found that inhibition of the JNK, p38 MAPK, PKCδ/θ, and Notch signal pathways affected HO-1 expression in keratinocytes. Considering the fact that the activation of these pathways is tightly connected to keratinocyte differentiation (47–50), the strict regulation of HO-1 may be critical for keratinocyte differentiation. Because the expression of epidermal proteins is precisely regulated in a temporal and spatial manner, HO-1 expression must be synchronized to that of other proteins during differentiation. However, HO-1 induction by Bach1 inactivation may be too rapid for differentiation and may be difficult to synchronize with the expression of other proteins.

Because Bach1 together with its competitor Nrf2 regulates cellular responses to oxidative stress, a fluctuation in the balance of these components may affect the pathological mechanisms underlying certain skin diseases. Interestingly, increased HO-1 expression is observed in psoriasis, a common skin disease marked by hyperproliferation and delayed keratinocyte differentiation (6). HO-1 expression seems to compensate for the abnormal features in psoriatic skin lesions, because HO-1 up-regulation attenuates keratinocyte hyperproliferation in an animal model of psoriasiform dermatitis (51). As high cell turnover occurs in psoriatic lesions, Bach1 may be inactivated by the increased concentrations of heme derived from degraded keratinocytes. Bach1 binds to heme through its heme regulatory motif, which results in the loss of its repressor activity (52) and the induction of HO-1. In addition, Bach1 has recently been reported to inhibit oxidative stress-induced cellular senescence by impeding the function of p53 (32). Bach1 function may affect

not only the pathogenesis of skin diseases but also the process of skin aging.

REFERENCES

1. Finkel, T. (2003) *Curr. Opin. Cell Biol.* **15,** 247–254
2. Briganti, S., and Picardo, M. (2003) *J. Eur. Acad. Dermatol. Venereol.* **17,** 663–669
3. Hanselmann, C., Mauch, C., and Werner, S. (2001) *Biochem. J.* **353,** 459–466
4. Vile, G. F., Basu-Modak, S., Waltner, C., and Tyrrell, R. M. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91,** 2607–2610
5. Wagener, F. A., van Beurden, H. E., von den Hoff, J. W., Adema, G. J., and Figdor, C. G. (2003) *Blood* **102,** 521–528
6. Wojas-Pelc, A., and Marcinkiewicz, J. (2007) *Int. J. Exp. Pathol.* **88,** 95–102
7. Igarashi, K., and Sun, J. (2006) *Antioxid. Redox. Signal.* **8,** 107–118
8. Oyake, T., Itoh, K., Motohashi, H., Hayashi, N., Hoshino, H., Nishizawa, M., Yamamoto, M., and Igarashi, K. (1996) *Mol. Cell. Biol.* **16,** 6083–6095
9. Hintze, K. J., Katoh, Y., Igarashi, K., and Theil, E. C. (2007) *J. Biol. Chem.* **282,** 34365–34371
10. Sun, J., Hoshino, H., Takaku, K., Nakajima, O., Muto, A., Suzuki, H., Tashiro, S., Takahashi, S., Shibahara, S., Alam, J., Taketo, M. M., Yamamoto, M., and Igarashi, K. (2002) *EMBO J.* **21,** 5216–5224
11. Ishikawa, M., Numazawa, S., and Yoshida, T. (2005) *Free Radic. Biol. Med.* **38,** 1344–1352
12. Suzuki, H., Tashiro, S., Sun, J., Doi, H., Satomi, S., and Igarashi, K. (2003) *J. Biol. Chem.* **278,** 49246–49253
13. Motohashi, H., and Yamamoto, M. (2007) *Cancer Sci.* **98,** 135–139
14. Shibahara, S. (2003) *Tohoku J. Exp. Med.* **200,** 167–186
15. Baranano, D. E., Rao, M., Ferris, C. D., and Snyder, S. H. (2002) *Proc. Natl. Acad. Sci. U.S.A.* **99,** 16093–16098
16. Otterbein, L. E., Soares, M. P., Yamashita, K., and Bach, F. H. (2003) *Trends Immunol.* **24,** 449–455
17. Numata, I., Okuyama, R., Memezawa, A., Ito, Y., Takeda, K., Furuyama, K., Shibahara, S., and Aiba, S. (2009) *J. Invest. Dermatol.* **129,** 2594–2603
18. Igarashi, K., Kataoka, K., Itoh, K., Hayashi, N., Nishizawa, M., and Yamamoto, M. (1994) *Nature* **367,** 568–572
19. Alam, J., Wicks, C., Stewart, D., Gong, P., Touchard, C., Otterbein, S., Choi, A. M., Burow, M. E., and Tou, J. (2000) *J. Biol. Chem.* **275,** 27694–27702
20. Ochiai, K., Katoh, Y., Ikura, T., Hoshikawa, Y., Noda, T., Karasuyama, H., Tashiro, S., Muto, A., and Igarashi, K. (2006) *J. Biol. Chem.* **281,** 38226–38234
21. Suzuki, H., Tashiro, S., Hira, S., Sun, J., Yamazaki, C., Zenke, Y., Ikeda-Saito, M., Yoshida, M., and Igarashi, K. (2004) *EMBO J.* **23,** 2544–2553
22. Okuyama, R., Ogawa, E., Nagoshi, H., Yabuki, M., Kurihara, A., Terui, T., Aiba, S., Obinata, M., Tagami, H., and Ikawa, S. (2007) *Oncogene* **26,** 4478–4488
23. Hennings, H., Michael, D., Cheng, C., Steinert, P., Holbrook, K., and Yuspa, S. H. (1980) *Cell* **19,** 245–254
24. Ogawa, E., Okuyama, R., Egawa, T., Nagoshi, H., Obinata, M., Tagami, H., Ikawa, S., and Aiba, S. (2008) *J. Biol. Chem.* **283,** 34241–34249
25. Sun, J., Brand, M., Zenke, Y., Tashiro, S., Groudine, M., and Igarashi, K. (2004) *Proc. Natl. Acad. Sci. U.S.A.* **101,** 1461–1466
26. Magnúsdóttir, E., Kalachikov, S., Mizukoshi, K., Savitsky, D., Ishida-Yamamoto, A., Panteleyev, A. A., and Calame, K. (2007) *Proc. Natl. Acad. Sci. U.S.A.* **104,** 14988–14993
27. Muto, A., Tashiro, S., Nakajima, O., Hoshino, H., Takahashi, S., Sakoda, E., Ikebe, D., Yamamoto, M., and Igarashi, K. (2004) *Nature* **429,** 566–571
28. Ochiai, K., Muto, A., Tanaka, H., Takahashi, S., and Igarashi, K. (2008) *Int. Immunol.* **20,** 453–460
29. Zenke-Kawasaki, Y., Dohi, Y., Katoh, Y., Ikura, T., Ikura, M., Asahara, T., Tokunaga, F., Iwai, K., and Igarashi, K. (2007) *Mol. Cell. Biol.* **27,** 6962–6971
30. Reichard, J. F., Sartor, M. A., and Puga, A. (2008) *J. Biol. Chem.* **283,** 22363–22370
31. Zhong, J. L., Raval, C., Edwards, G. P., and Tyrrell, R. M. (2010) *Free Radic. Biol. Med.* **48,** 196–206
32. Dohi, Y., Ikura, T., Hoshikawa, Y., Katoh, Y., Ota, K., Nakanome, A., Muto, A., Omura, S., Ohta, T., Ito, A., Yoshida, M., Noda, T., and Igarashi, K. (2008) *Nat. Struct. Mol. Biol.* **15,** 1246–1254
33. Takahashi, T., Shimizu, H., Morimatsu, H., Inoue, K., Akagi, R., Morita, K., and Sassa, S. (2007) *Mini Rev. Med. Chem.* **7,** 745–753
34. Omura, S., Suzuki, H., Toyofuku, M., Ozono, R., Kohno, N., and Igarashi, K. (2005) *Genes Cells* **10,** 277–285
35. Yano, Y., Ozono, R., Oishi, Y., Kambe, M., Yoshizumi, M., Ishida, T., Omura, S., Oshima, T., and Igarashi, K. (2006) *Genes Cells* **11,** 791–803
36. Kanno, H., Ozawa, H., Dohi, Y., Sekiguchi, A., Igarashi, K., and Itoi, E. (2009) *J. Neurotrauma* **26,** 31–39
37. Duckers, H. J., Boehm, M., True, A. L., Yet, S. F., San, H., Park, J. L., Clinton Webb, R., Lee, M. E., Nabel, G. J., and Nabel, E. G. (2001) *Nat. Med.* **7,** 693–698
38. Odaka, Y., Takahashi, T., Yamasaki, A., Suzuki, T., Fujiwara, T., Yamada, T., Hirakawa, M., Fujita, H., Ohmori, E., and Akagi, R. (2000) *Biochem. Pharmacol.* **59,** 871–880
39. Poss, K. D., and Tonegawa, S. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94,** 10925–10930
40. Soares, M. P., Lin, Y., Anrather, J., Csizmadia, E., Takigami, K., Sato, K., Grey, S. T., Colvin, R. B., Choi, A. M., Poss, K. D., and Bach, F. H. (1998) *Nat. Med.* **4,** 1073–1077
41. Yachie, A., Niida, Y., Wada, T., Igarashi, N., Kaneda, H., Toma, T., Ohta, K., Kasahara, Y., and Koizumi, S. (1999) *J. Clin. Invest.* **103,** 129–135
42. Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., Oyake, T., Hayashi, N., Satoh, K., Hatayama, I., Yamamoto, M., and Nabeshima, Y. (1997) *Biochem. Biophys. Res. Commun.* **236,** 313–322
43. Kawachi, Y., Xu, X., Taguchi, S., Sakurai, H., Nakamura, Y., Ishii, Y., Fujisawa, Y., Furuta, J., Takahashi, T., Itoh, K., Yamamoto, M., Yamazaki, F., and Otsuka, F. (2008) *J. Invest. Dermatol.* **128,** 1773–1779
44. Khor, T. O., Huang, M. T., Kwon, K. H., Chan, J. Y., Reddy, B. S., and Kong, A. N. (2006) *Cancer Res.* **66,** 11580–11584
45. Wakabayashi, N., Itoh, K., Wakabayashi, J., Motohashi, H., Noda, S., Takahashi, S., Imakado, S., Kotsuji, T., Otsuka, F., Roop, D. R., Harada, T., Engel, J. D., and Yamamoto, M. (2003) *Nat. Genet.* **35,** 238–245
46. Fuchs, E. (2008) *J. Cell Biol.* **180,** 273–284
47. Okuyama, R., Tagami, H., and Aiba, S. (2008) *J. Dermatol. Sci.* **49,** 187–194
48. Dotto, G. P. (1999) *Crit. Rev. Oral Biol. Med.* **10,** 442–457
49. Zenz, R., and Wagner, E. F. (2006) *Int. J. Biochem. Cell Biol.* **38,** 1043–1049
50. Eckert, R. L., Efimova, T., Balasubramanian, S., Crish, J. F., Bone, F., and Dashti, S. (2003) *J. Invest. Dermatol.* **120,** 823–828
51. Ma, L. J., You, Y., Bai, B. X., and Li, Y. Z. (2009) *Arch. Dermatol. Res.* **301,** 459–466
52. Ogawa, K., Sun, J., Taketani, S., Nakajima, O., Nishitani, C., Sassa, S., Hayashi, N., Yamamoto, M., Shibahara, S., Fujita, H., and Igarashi, K. (2001) *EMBO J.* **20,** 2835–2843