Genetic ablation of Bach1 gene reduces hyperoxic lung injury in mice: role of IL-6

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Abstract—Bach1 is a transcriptional repressor of heme oxygenase (HO)-1 gene. *Bach1*-null (*Bach1*$^{-/-}$) mice are reported to be protected from myocardial ischemia/reperfusion injury; however, the effect of *Bach1*-disruption on another oxidative stress model of hyperoxic lung injury has yet to be determined. To investigate the role of Bach1 in hyperoxic lung injury, *Bach1*$^{-/-}$ mice and wild type (WT) mice were exposed to 90% O_2. During hyperoxic exposure, the survival of *Bach1*$^{-/-}$ mice was significantly longer than that of WT mice. However, the administration of zincprotoporphyrin, an inhibitor of HO-1 activity, did not change the mortality in either of the mice, thus suggesting that this protective effect was not mediated by an HO-1 overexpression in *Bach1*$^{-/-}$ mice. The indices of lung injury in the lungs of *Bach1*$^{-/-}$ mice were lower than those of WT mice; unexpectedly, however, the levels of IL-6 in bronchoalveolar lavage (BAL) fluid from *Bach1*$^{-/-}$ mice were significantly higher than those of WT mice. Interestingly, the intrapulmonary administration of small interfering RNA against IL-6 was shown to reduce the IL-6 levels in BAL fluids and shorten the survival in*Bach1*$^{-/-}$ mice during hyperoxic exposure. In addition, a chromatin immunoprecipitation analysis revealed the binding of Bach1 to the IL-6 promoter and its detachment following oxidative stress. Considering the previous observation that the transgenic mice overexpressing IL-6 are protected from hyperoxic lung injury, these results therefore indicate that IL-6 mediates an increased survival in *Bach1*$^{-/-}$ mice during hyperoxic exposure.

Key words: Bach1; heme oxygenase-1; hyperoxic lung injury; IL-6; oxidative stress; survival; bronchoalveolar lavage; small interfering RNA; chromatin immunoprecipitation analysis

INTRODUCTION

Hyperoxic lung injury is caused by the prolonged administration of fractional inspired concentrations of oxygen greater than 50 to 60%. The pathophysiology of this pulmonary toxicity has been previously characterized in animal models [1-4]. These studies demonstrated that the toxic concentrations of O_2 generate oxygen-derived free radicals that damage lung epithelial and endothelial cells, thus leading to a protein-rich fluid that floods the alveolar space. Recent studies have also demonstrated that this injury is associated with a cell-death response with features of both cell necrosis and apoptosis [5, 6]. In fact, exposure to hyperoxia (>90% O_2) for more than three days is reported to be fatal for animals [7].

Heme oxygenase (HO)-1 has been shown to be induced in the lung during exposure to hyperoxia [8]. Heme oxygenase catalyzes the degradation of heme to carbon monoxide (CO), biliverdin, and iron [9, 10]. There are two isoforms of HO that are products of distinct genes and differ in tissue distribution and regulation. HO-1 is the highly inducible enzyme that is induced by oxidative stress [11], agents such as heme [12], heat shock [13], IL-1, TNF-α [14], and IL-6 [15], whereas HO-2 is a housekeeping enzyme that is expressed constitutively in almost all tissues [16]. CO, biliverdin and its metabolite bilirubin, have been shown to have antioxidative, anti-inflammatory, and antiapoptotic effects on cells [17-19]; however, these substances also show cytotoxicity when their quantities increase. Iron catalyzes free radical reactions; therefore, it can accelerate oxygen toxicity [20, 21]. However, it is also believed to not cause any serious toxicity because the iron is trapped in the central cavity of ferritin nanocages [22]. Following these observations, the role of HO in hyperoxic lung injury is thus considered to be complex. The mice overexpressing HO-1 in the lungs have been reported to be protected from hyperoxic lung injury [23, 24]. On the other hand, the mice lacking HO-

1 gene are also protected from hyperoxic lung injury [20], and the mice deficient in HO-2 gene, in which HO-1 is excessively induced, therefore show a worse degree of hyperoxic lung injury than that observed in the WT mice [25].

Bach1 is a transcriptional repressor of HO-1 gene (*Hmox-1*) and β-globin gene [26, 27]. The enhancers of *Hmox-1* carry multiple Maf-recognition elements (MAREs). Heterodimer of the small Maf protein and NF-E2-related factor2 (Nrf2) activate *Hmox-1* through binding to MAREs. In contrast, the heterodimer of small Maf protein and Bach1 repress MARE-dependent transcription. The ability of Nrf2 to activate HO-1 expression is greatly reduced in the presence of Bach1 under normal conditions [27, 28]. In various stress situations (inflammation, oxidative stress, heme, cadmium, etc.), Bach1 detaches from and Nrf2/small Maf binds to MAREs, and *Hmox-1* is activated and HO-1 is transcribed [29, 30] In *Bach1*-null (*Bach1*$^{-/-}$) mice, the transcription of HO-1 is constitutively up-regulated, thus leading to increased levels of protein and enzymatic activity under normal conditions in several organs such as heart, lung and liver [27]. Previous studies have shown that *Bach1*$^{-/-}$ mice are protected from oxidative stress such as atherosclerosis and myocardial ischemia-reperfusion injury [31, 32] and mice deficient in Nrf2 gene are more susceptible to hyperoxic lung injury, another oxidative stress model [33]. The underlining mechanism of the protection of *Bach1*$^{-/-}$ mice from oxidative stress can be explained, in part, by the up-regulation of HO-1 activity [31], however, much remains to be elucidated.

To further investigate the role of Bach1 in oxidative stress, *Bach1*$^{-/-}$ mice were used in a model of hyperoxic lung injury to evaluate the survival of the mice, the association with the HO-1 activity, and the indices of lung tissue injury.

MATERIALS AND METHODS

Animals

Bach1$^{-/-}$ mice, originally produced by Sun et al. [27], were repeatedly backcrossed with C57BL/6 mice at least up to 12 generations and maintained in the animal facility of Hiroshima University. Age-, weight-, and gender-matched C57BL/6 wild type (WT) mice (*Bach1*$^{+/+}$) were used as control groups. All experimental procedures were approved and carried out in accordance with the Guidelines of Hiroshima University Graduate School of Biomedical Sciences.

Oxygen exposure

The mice were exposed to hyperoxia (90 ± 2%) in a sealed 30-liter chamber (Allentown, Inc., NJ, USA). Food and water were provided ad libitum. Humidified oxygen, provided by an oxygen concentrator (TO-90-5H; TEIJIN, Tokyo, Japan) was delivered to the chamber to provide 8 changes/h (4 liters/min flow rate). The O2 concentration in the chamber was continuously monitored and kept at 90 ± 2% throughout the experiments.

Administration of zincprotoporphyrin (ZnPP)

To block the HO activity, ZnPP (Sigma-Aldrich Corp. St. Louis, MO, USA) was injected intraperitoneally as previously described with slight modifications [32]. Briefly, ZnPP was dissolved in saline with 0.1 N NaOH and neutralized with 0.1 N HCl to reach pH 7.4 immediately before administration. The injection of ZnPP (10 mg/kg) was started 24 h prior to hyperoxic exposure and, thereafter, it was administered once daily until 48 h after the start of hyperoxic exposure.

Bronchoalveolar lavage (BAL)

The mice were sacrificed with a lethal dose of pentobarbital. The trachea was cannulated with an 18-gauge needle, and the lungs were lavaged twice with 1 ml of PBS. The lavage fluids were pooled and were centrifuged at 500 g for 10 min at 4°C. The supernatants were stored for the measurement of IL-6 and TNF-α concentrations using ELISA kits obtained from R&D Systems (Minneapolis, MN, USA). The cell pellets were resuspended in 1 ml of Dulbecco's modified Eagle's medium, and the total cell numbers were counted with a hemocytometer. Differential cell counts were determined by counting at least 300 cells on a smear prepared using a cytospin at 600 rpm for 5 minutes (Shandon Inc., Pittsburgh, Pennsylvania, USA) and stained with Diff-Quick (Kokusai Shiyaku, Kobe, Japan).

Histological examination

Lungs were inflated with 10% neutral buffered formalin and the trachea was ligated. The heart and lungs were removed, fixed in 4% formalin, and embedded in paraffin. Hematoxylin and eosin stains were done on 4 μ sections prepared from the blocks.

Immunohistochemistry

Formalin-fixed tissue sections were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. Antigen retrieval was performed by boiling the sections in 0.1 M citrate buffer (pH 6.0). After endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol for 10 min and non-specific protein binding was blocked in normal goat serum for 20 min, the sections were incubated with polyclonal rabbit anti-mouse ssDNA antibody (Dako, Glostrup, Denmark) diluted at the ratio of 1:1500 in PBS or with polyclonal rabbit anti-mouse IL-6 antibody (GeneTex, Inc., San Antonio, Texas, USA) diluted at the ratio of 1:1000 in PBS at 4°C overnight.

After washed three times with PBS, bound antibody was detected using a VECTASTAIN Elite ABC kit (Vector Laboratories Inc., Burlingame, CA, USA) and DAB+ (3 3'-Diaminobenzidine Tetrahydrochloride) Liquid System (Dako, Glostrup, Denmark) as a peroxidase substrate. Thereafter, the sections were counterstained with hematoxylin. The apoptotic index was defined as the ratio of cells stained with the antibody against ssDNA to the total number of the cells based on the counting at least 200 cells per field (x400) out of 10 independent fields under light microscopy.

Determination of HO-1 and HO-2 levels in lung

The lung tissue specimens were homogenized on ice in 2.0 ml of cold PBS and centrifuged 10,000 g for 10 min at 4°C. The supernatants were prepared and HO-1 level was determined using an HO-1 ELISA kit (TAKARA bio, Tokyo, Japan) according to the manufacturer's protocol. The level of HO-2 in lung was assessed using a Western blot analysis. Briefly, the supernatants of lung homogenates were mixed 2:1 with triple-strength Lammelli sample buffer (200 mM Tris/HCl, 9.6% SDS, 30% glycerol, 0.016% bromphenol blue, and 6% 2-mercaptoethanol), and boiled for 5 min. SDS–PAGE was performed and the PAGE-separated proteins were electroblotted onto nitrocellulose membranes (GE Healthcare Bio-sciences, Piscataway, NJ) and incubated with rabbit anti-HO-2 polyclonal antibody (Stressgen Biotechnologies Corp., Victoria, BC, Canada). After washed in WB Stripping Solution (nacalai tesque, Kyoto, Japan), the membranes were incubated with a goat anti-rabbit IgG-HRP antibody (GE Healthcare Bio-sciences, Piscataway, NJ, USA). The protein bands were imaged using an ECL Western-blotting analysis system (GE Healthcare Bio-sciences, Piscataway, NJ, USA). The intensity of each protein band was quantitated using the Image-J software program (NIH, Bethesda, Maryland, USA).

Protein concentration

The protein concentrations in samples were determined using a BCA Protein Assay Kit (PIERCE, Rockford, IL, USA) according to the manufacturer's protocol.

Inhibition of IL-6 expression in the lungs using small interfering RNA (siRNA)

SiRNA targeting mouse IL-6 (IL-6 siRNA) and non-silencing siRNA (NS siRNA) as a control were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Two hours before the start of hyperoxic exposure, 500 pmol of IL-6 siRNA or NS siRNA dissolved in 50 μl of PBS were intratracheally administered to the mice. To confirm the inhibition of IL-6 expression in the lungs, some of the mice from each group were sacrificed after 72 h of hyperoxic exposure and the levels of IL-6 in BALF were thus determined as described above.

Chromatin immunoprecipitation (ChIP) analysis

A ChIP analysis was performed using the ChIP-IT Express kit (Active Motif, Carlsbad, California) following the manufacturer's protocol. In brief, Lewis lung carcinoma (LLC) cells and murine macrophage RAW264 cells were incubated in the presence or absence of H_2O_2 (the concentration were 0.3 mM for LLC and 0.1 mM for RAW264) as an oxidative stress for 3 hr, and then fixed with formaldehyde for 10 min. The cells were lysed, sheared using Enzymatic Shearing kit, and incubated overnight with anti-Bach1 antibody (H-130) or normal rabbit IgG (negative control) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.). Precipitated DNA were analyzed by PCR (40 cycles) using ChIP-qPCR Primer for mouse IL-6 promoter (NM_031168.1 (-) 01kb, SuperArray Bioscience Corporation, Frederick, MD, U.S.A.)

Statistical analysis

The data are presented as the mean ± SEM. Student's *t*-test was performed for comparison between two groups (*Bach1*$^{-/-}$ mice and WT mice). A one-way ANOVA was performed for comparison among six groups of HO-2 protein levels. Kaplan-Meier curves were used to estimate survival and were compared with the use of the log-rank test. Differences were considered significant when the *P* value was <0.05. Descriptive statistical analyses were performed with the Statcel2 software program (OMS publishing Inc., Saitama, Japan).

RESULTS

Expression of HO-1 and HO-2 in the lungs of Bach1$^{-/-}$ mice during hyperoxic exposure

To investigate the expression of HO-1 and HO-2 in the lungs of *Bach1*$^{-/-}$ mice and WT mice during hyperoxic exposure, the protein levels of these HO in the lungs were evaluated as described in the Materials and Methods. As shown in Fig. 1A, the HO-1 level in *Bach1*$^{-/-}$ mice lungs were significantly higher than that of WT mice even prior to hyperoxic exposure, and the time-dependent increase of HO-1 expression in *Bach1*$^{-/-}$ mice lungs was much greater than that of WT mice during hyperoxic exposure. In contrast, the expression levels of HO-2 in the lungs of both *Bach1*$^{-/-}$ and WT mice was found to not be influenced by hyperoxic exposure (Fig. 1B).

Survival analysis during hyperoxic exposure

To determine whether *Bach1* disruption influences the degree of oxygen toxicity, the survival of *Bach1*$^{-/-}$ mice was compared with that of WT mice during hyperoxic exposure. As shown in Fig. 2, the *Bach1*$^{-/-}$ mice survived significantly longer than the WT mice. To identify the association of HO-1 activity in hyperoxic lung injury, subgroups of *Bach1*$^{-/-}$ and WT mice were intraperitoneally administered with ZnPP, an inhibitor of HO-1 activity, during hyperoxic exposure. Interestingly, the administration of ZnPP did not significantly change the mortality in either group of mice (Fig. 2).

Degree of lung injury in hyperoxic exposure

To determine whether there is a difference in the degree of hyperoxic lung injury between *Bach1*$^{-/-}$ mice and WT mice, the microscopic features of the lungs and the protein concentrations in the bronchoalveolar lavage fluid (BALF) from *Bach1*$^{-/-}$ mice

and WT mice were compared before and after hyperoxic exposure. As shown in Fig. 3, diffuse congestion and interstitial edema were observed in the lungs of both *Bach1*$^{-/-}$ and WT mice but the severity of these conditions were much less in *Bach1*$^{-/-}$ mice. Regarding the protein concentration in BALF, which reflects protein leakage into the alveolar space, it was found to be significantly higher in the WT mice in comparison to *Bach1*$^{-/-}$ mice 90 h after the start of hyperoxic exposure (Fig. 4A). In addition, the administration of ZnPP did not affect the degree of lung injury as assessed by microscopic observations of the lung and the protein level in BALF (data not shown).

Effect of Bach1-disruption on inflammatory indices in BALF

To evaluate the inflammatory response in the lung during hyperoxic exposure, the numbers of inflammatory cells was counted and the inflammatory cytokines, IL-6 and TNF-α, in BALF obtained from *Bach1*$^{-/-}$ mice and WT mice were measured. Because lymphocytes and eosinophils were scarcely observed in the BALF obtained from the mice exposed to hyperoxia (data not shown), the counts of macrophages and neutrophils are thus presented. Fig. 4B shows that, prior to hyperoxic exposure, the number of macrophages in BALF of *Bach1*$^{-/-}$ mice was already significantly higher than that of WT mice. In both mice, the numbers of macrophages and neutrophils in BALF increased after 48 h of hyperoxic exposure. Unexpectedly, however, the number of inflammatory cells in BALF from *Bach1*$^{-/-}$ mice was significantly higher than that of WT mice. Specifically, there was a big difference in the neutrophil count in BALF between *Bach1*$^{-/-}$ mice and WT mice after 72 and 90 h of hyperoxic exposure (Fig. 4C). No significant difference was observed in the TNF-α levels in BALF between the *Bach1*$^{-/-}$ mice and the WT mice (Fig. 5A). However, the IL-6 level in BALF obtained

from *Bach1*$^{-/-}$ mice was found to be significantly higher than that of WT mice after 24, 48 and 72 h of hyperoxic exposure (Fig. 5B).

Determination of apoptosis in hyperoxic lung injury

Hyperoxic lung injury is characterized by the cell-death response characteristic of apoptosis [5]. To assess the degree of apoptosis in lung tissue, the lung sections were immunostained with a polyclonal antibody against ssDNA. As shown in Fig. 6 A and B, nuclear staining in alveolar epithelial cells was observed in both *Bach1*$^{-/-}$ and WT mice; however, the stained cells were apparently more scarce in the *Bach1*$^{-/-}$ mice. Further supporting this observation, the apoptotic index in *Bach1*$^{-/-}$ mice was significantly lower in comparison to that of the WT mice after 48 h of exposure to 90% O_2 (Fig 6C).

Immunohistochemical detection of IL-6 in lung

To characterize the cellular source of IL-6 in lungs exposed to hyperoxia, immunohistochemical studies were carried out on the lung sections obtained from *Bach1*$^{-/-}$ and WT mice. IL-6 was detected in the bronchial and alveolar epithelial cells in both mice, however, the staining intensity in the *Bach1*$^{-/-}$ mice was apparently higher than that in WT mice at 48 h of exposure to 90% O_2 (Figs. 7C and D). After 90 h of hyperoxic exposure, there was no difference in the staining intensity between *Bach1*$^{-/-}$ mice and WT mice (Figs. 7E and 7F). These observations were compatible with the levels of IL-6 in BALF.

Effect of the intrapulmonary administration of IL-6 siRNA in Bach1$^{-/-}$ *mice*

To determine the association between IL-6 and hyperoxic lung injury in *Bach1*$^{-/-}$ mice, IL-6 siRNA and NS siRNA were intratracheally administered to the *Bach1*$^{-/-}$ mice before the start of hyperoxic exposure, and a survival analysis and the measurement of the levels of IL-6 in BALF were performed during hyperoxic exposure. As shown in Fig. 8A, the intrapulmonary administration of IL-6 siRNA significantly reduced the IL-6 level in BALF obtained from *Bach1*$^{-/-}$ mice after 72 h of hyperoxic exposure in comparison to that of the mice administered NS siRNA. Furthermore, the *Bach1*$^{-/-}$ mice which were intratracheally administered IL-6 siRNA were observed to survive significantly shorter than those administered NS siRNA (Fig. 8B).

ChIP analysis

To determine whether Bach1 interacts with the IL-6 gene, a ChIP analysis was performed on LLC and RAW264 cells either with or without stimulation by the induction of oxidative stress. As shown in Fig. 9, Bach1 was found to be bound to the IL-6 promoter and thereafter it became detached after stimulation of the cells with H_2O_2.

DISCUSSION

The present study revealed that mice lacking Bach1 gene are relatively well protected against hyperoxic lung injury. This protection in *Bach1*$^{-/-}$ mice was demonstrated by the significantly better survival, the lesser tissue injury according to the findings of a histological examination, the smaller amount of protein in BALF, and the lesser extent of apoptosis, in comparison to the WT mice. Unexpectedly, however, the number of macrophages and neutrophils in BALF obtained from *Bach1*$^{-/-}$ mice was significantly higher than those of WT mice after exposure to hyperoxia. Similarly, the levels of IL-6 but not TNF-α in the BALF of *Bach1*$^{-/-}$ mice induced by hyperoxia were significantly higher in comparison to those in the WT mice. In addition, the intrapulmonary administration of IL-6 siRNA was shown to decrease the IL-6 levels in BALF and shorten the survival in *Bach1*$^{-/-}$ mice during hyperoxic exposure. Furthermore, a ChIP analysis performed on LLC and RAW246 cells demonstrated both the direct binding of Bach1 to the IL-6 promoter and its detachment from this region following the stimulation of the cells by oxidative stress.

The protective effect against hyperoxic lung injury observed in *Bach1*$^{-/-}$ mice was thought to be achieved by the anti-oxidant activity of HO-1 that was strongly induced in those mice. However, previous studies have demonstrated that the involvement of HO-1 in hyperoxic lung injury is complicated. Lung specific-induction of HO-1 by gene transfer [23] or hemoglobin [24] appears to protect lungs from injury caused by exposure to hyperoxia. In hyperoxic exposure, on the other hand, mice deficient in HO-2 gene, whose HO-1 in the lung was induced two or three times higher than that of WT mice, show shorter survival and more severer lung injury than those of WT mice [25]. Furthermore, mice lacking HO-1 gene show a longer survival and a lesser extent of lung

injury than those of the WT mice during hyperoxic exposure, and this relative protection from hyperoxic lung injury is reversed by the transduction of human HO-1 [20]. In the present study, *Bach1*$^{-/-}$ mice expressing more HO-1 than WT mice during hyperoxic exposure were relatively protected from hyperoxic lung injury, however, this protection was not reversed by the administration of ZnPP, an inhibitor of HO-1 activity. Considering the uncertainties of the protective effect of HO-1 on hyperoxic lung injury, it is possible that HO-1 has little effect on the protection from hyperoxic lung injury in *Bach1*$^{-/-}$ mice.

During hyperoxic exposure, a higher level of IL-6 was found to be induced in the lung of *Bach1*$^{-/-}$ mice in comparison to WT mice and, interestingly, the intrapulmonary administration of IL-6 siRNA was observed to decrease the IL-6 levels in BALF and shorten the survival in *Bach1*$^{-/-}$ mice. These data strongly suggest that IL-6 mediated an increased survival in *Bach1*$^{-/-}$ mice during hyperoxic exposure. In fact, the protective role of IL-6 on hyperoxic lung injury has been firmly established by a previous study which demonstrated that transgenic mice overexpressing IL-6 in the lungs are protected from hyperoxic acute lung injury [34]. In this report, the IL-6 transgenic mice lived more than 8 days longer than WT mice during chronic hyperoxic exposure and IL-6 was shown to work as anti-apoptotic factor in hyperoxic lung injury. In the present study, the apoptosis in the lungs of *Bach1*$^{-/-}$ mice induced by hyperoxic exposure also decreased, however, the survival of *Bach1*$^{-/-}$ mice was extended by only one day in comparison to that of WT mice. The shorter survival of *Bach1*$^{-/-}$ mice in comparison to that of IL-6 transgenic mice during chronic exposure to hyperoxia may be explained by the difference in the IL-6 expression pattern between *Bach1*$^{-/-}$ mice and IL-6 transgenic mice. Although, in IL-6 transgenic mice, IL-6 is overexpressed in the lung before the start of hyperoxic exposure, IL-6 is induced in the lungs of *Bach1*$^{-/-}$ mice following

hyperoxic exposure and the levels are less than half of the IL-6 transgenic mice. Therefore, the protection of the lungs from hyperoxic lung injury due to IL-6 may be delayed and slight in *Bach1*$^{-/-}$ mice in comparison to IL-6 transgenic mice. Another possibility is the influence of HO-1 highly induced in the lungs of *Bach1*$^{-/-}$ mice. Suttner et al. demonstrated that, despite cytoprotection with a low (less than five-fold) HO activity, high levels of HO-1 expression (greater than 15-fold) in cells are associated with a significant degree of oxygen cytotoxicity [21]. In *Bach1*$^{-/-}$ mice, a greater than 15-fold HO-1 expression in the lungs in comparison to that in WT mice was observed after 48 h of hyperoxic exposure. Therefore, excessive levels of HO-1 activity in *Bach1*$^{-/-}$ mice might neutralize the protective effect of IL-6 in hyperoxic exposure.

Despite the lesser extent of lung injury observed in histological examination, the number of inflammatory cells in BALF obtained from *Bach1*$^{-/-}$ mice was higher than that of WT mice in hyperoxic exposure. These higher cell counts of macrophages and neutrophils in BALF may also be explained by IL-6. IL-6 is reported to promote the differentiation of monocytes to macrophages [35] and the migration of neutrophils [36]. These observations support the fact that a larger number of macrophages and neutrophils migrate to the lungs of *Bach1*$^{-/-}$ mice following hyperoxic exposure. There is no good explanation for the observation that the macrophage count in BALF from *Bach1*$^{-/-}$ mice was larger than that of Wt mice before hyperoxic exposure. The ablation of Bach1 gene may therefore be related with this phenomenon; however, further investigation is needed.

A novel finding in the present study is that both the direct binding of Bach1 to the IL-6 promoter and its detachment from this region following the stimulation of the cells with an oxidative stress were shown by a ChIP analysis. Considering that a significantly increased expression of IL-6 was observed in *Bach1*$^{-/-}$ mice at the early phase of

hyperoxic lung injury, these results strongly suggest that IL-6 is a direct target gene of Bach1 and Bach1 may therefore be a transcriptional repressor of IL-6 gene. In addition, the strong induction of IL-6 expression observed in WT mice at the late phase of hyperoxic lung injury may be explained by this association between Bach1 and the IL-6 gene. As shown in the present study and the previous reports [37, 38], the IL-6 expression in the lungs of WT mice sharply rises at the timing close to death during hyperoxic exposure. Judging from the rapid increase of the IL-6 expression in *Bach1*$^{-/-}$ mice soon after the start of hyperoxic exposure, we can speculate that the binding of Bach1 to the IL-6 promoter to repress the expression is tight and its detachment from this region thus requires a sufficient amount and duration of hyperoxic stress. There have been a few previous reports describing the relationship between HO-1 and IL-6. CO and biliverdin, degradation products of heme by HO-1, are shown to inhibit the production of IL-6 [39, 40]. In contrast, one report demonstrated that HO-1 induces the production of IL-6 and TNF-α through the production of CO and cyclic GMP [41]. In the present study, the IL-6 levels in BALF of *Bach1*$^{-/-}$ mice after hyperoxic exposure increased independently of those of TNF-α, thus again suggesting that Bach1 is associated with the induction of IL-6 through pathways independent of HO-1.

In conclusion, *Bach1*$^{-/-}$ mice were relatively well protected from the injury induced by hyperoxic exposure. This protective effect did not appear to be mediated by the HO-1 overexpression in *Bach1*$^{-/-}$ mice but by IL-6 induced in the lungs of *Bach1*$^{-/-}$ mice following hyperoxic exposure. The involvement of IL-6 in the protective effect on hyperoxic lung injury was confirmed by the experiment in which IL-6 siRNA was intratracheally administered to *Bach1*$^{-/-}$ mice. Furthermore, a ChIP analysis demonstrated the direct binding of Bach1 to the IL-6 promoter and its detachment following the stimulation of the cells with oxidative stress. The interactions among

Bach1, HO-1, and IL-6 in hyperoxic lung injury proposed by the results of the present study are summarized in Fig. 10. Based on these results, we therefore conclude that Bach1 is a transcriptional repressor of IL-6 gene and increased expression of IL-6 in *Bach1*$^{-/-}$ mice may thus lead to an improved survival during hyperoxic exposure.

List of Abbreviations: HO, heme oxygenase; Bach1-/-, Bach1-null; WT, wild type; CO, carbon monoxide; Hmox-1, HO-1 gene; MAREs, Maf-recognition elements; NRF2, NF-E2-related factor2; ZnPP, zincprotoporphyrin; BAL, bronchoalveolar lavage; siRNA, small interfering RNA; ChIP, chromatin immunoprecipitation; LLC, Lewis lung carcinoma; BALF, bronchoalveolar lavage fluid.

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Figure legends

Fig. 1. Lung HO-1 and HO-2 protein levels. (A) The protein levels of HO-1 in the lungs of *Bach1*$^{-/-}$ mice and WT mice. The lungs were excised from WT mice (open bars) and *Bach1*$^{-/-}$ mice (solid bars) at baseline and after 24 h, 48 h, 72 h, and 90 h of exposure to 90% O_2. The protein levels of HO-1 in the lungs were measured as described in the Methods. The data are shown as the mean ± SEM of at least 5 animals in each group (*$P < 0.01$ versus WT mice at the same time point). (B) The protein levels of HO-2 in the lungs of *Bach1*$^{-/-}$ mice and WT mice. The lungs were excised from WT mice (open bars) and *Bach1*$^{-/-}$ mice (solid bars) at baseline and after 48 h and 90 h of exposure to 90% O_2. Protein levels of HO-2 in the lungs were measured by a Western blot analysis. The protein bands were quantitated using Image-J software (NIH, Bethesda, Maryland, USA) and then were normalized to the β-actin levels. The data are shown as the mean ± SEM of 3 mice in each group. There were no significant differences in the levels of HO-2 between the six groups.

Fig. 2. Survival of *Bach1*$^{-/-}$ and WT mice following hyperoxic exposure. Six mice from each group were exposed to 90% O_2 until all animals had died. Open circles = WT mice; open triangles = WT mice injected with ZnPP; open squares = *Bach1*$^{-/-}$ mice; closed circles = *Bach1* $^{-/-}$ mice injected with ZnPP. $P = 0.017$: *Bach1*$^{-/-}$ mice versus WT mice.

Fig. 3. A histological analysis of the mouse lung before and after hyperoxic exposure. Formalin-fixed sections of mice lungs were stained with hematoxylin and eosin. (A)

WT mice at baseline. (B) *Bach1*$^{-/-}$ mice at baseline. (C) WT mice after 48 h of hyperoxic exposure. (D) *Bach1*$^{-/-}$ mice after 48 h of hyperoxic exposure. (E) WT mice after 90 h of hyperoxic exposure. (F) *Bach1*$^{-/-}$ mice after 90 h of hyperoxic exposure. Scale bars: 200 μm.

Fig. 4. Analysis of BALF. (A) The protein levels in BALF of *Bach1*$^{-/-}$ mice and WT mice. BAL was performed on WT mice (open bars) and *Bach1*$^{-/-}$ mice (solid bars) at baseline and after 24 h, 48 h, 72 h, and 90 h of exposure to 90% O_2. The levels of protein were assessed as described in the Methods. The data are shown as the mean ± SEM of 5 mice in each group. *$P < 0.05$ versus WT mice at the same time point. (B) Numbers of macrophages and (C) neutrophils in BALF of WT mice and *Bach1*$^{-/-}$ mice before and after hyperoxic exposure. BAL was performed on WT mice (open bars) and *Bach1*$^{-/-}$ mice (solid bars) as described in the Methods. The data are shown as the mean ± SEM of 5 mice in each group. *$P < 0.01$ versus WT mice at the same time point. †$P < 0.05$ versus WT mice at the same time point.

Fig. 5. The inflammatory cytokines in BALF. (A) The TNF-α.levels in BALF of WT mice (open bars) and *Bach1*$^{-/-}$ mice (solid bars). There were no significant differences in the levels of TNF-α between *Bach1*$^{-/-}$ mice and the time matched WT mice. (B) The IL-6 levels in BALF of WT mice (open bars) and *Bach1*$^{-/-}$ mice (solid bars). *$P < 0.01$ versus WT mice at the same time point. †$P < 0.05$ versus WT mice at the same time point. The data are shown as the mean ± SEM of at least 5 animals in each group.

Fig. 6. Detection of apoptosis in mice lungs after 48 h of hyperoxic exposure. Formalin-fixed sections of mice lungs were immunostained with anti-ssDNA antibody as described in the Methods. (A) WT mice after 48 h of hyperoxic exposure. (B) *Bach1*$^{-/-}$ mice after 48 h of hyperoxic exposure. Scale bars: 50 μm. (C) Apoptotic index in lung tissue specimens of WT (open bar) and *Bach1*$^{-/-}$ (solid bar) mice after 48 h of hyperoxic exposure. The apoptotic index was calculated as described in the Methods. **P* < 0.01 versus WT mice. The data are shown as the mean ± SEM.

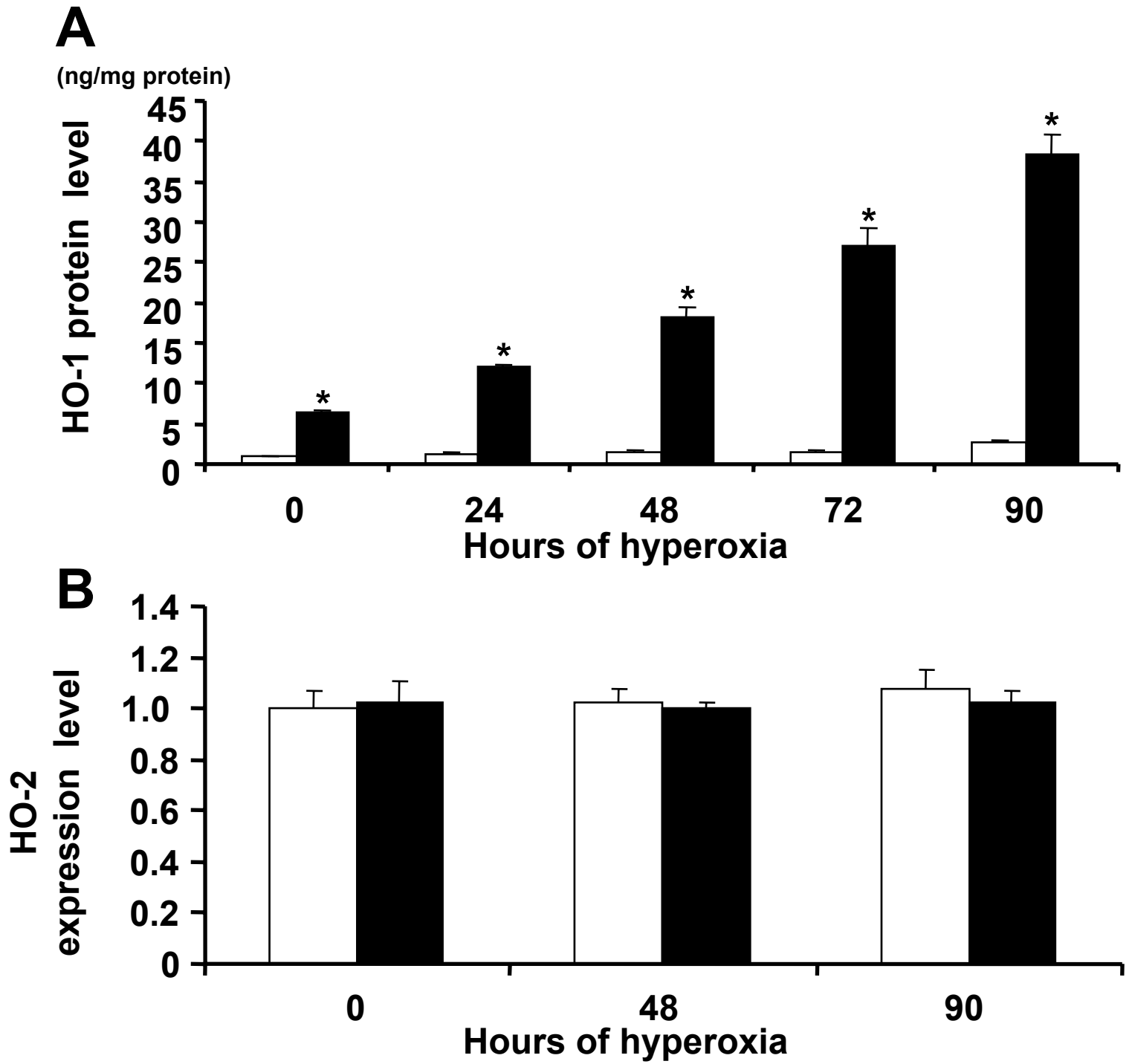
Fig. 7. Detection of IL-6 in mice lung before and after hyperoxic exposure. Formalin-fixed sections of mice lungs were analyzed for IL-6 expression by immunostaining as described in the Methods. (A) WT mice at baseline. (B) *Bach1*$^{-/-}$ mice at baseline. (C) WT mice after 48 h of hyperoxic exposure. (D) *Bach1*$^{-/-}$ mice after 48 h of hyperoxic exposure. (E) WT mice after 90 h of hyperoxic exposure. (F) *Bach1*$^{-/-}$ mice after 90 h of hyperoxic exposure. Scale bars: 50 μm.

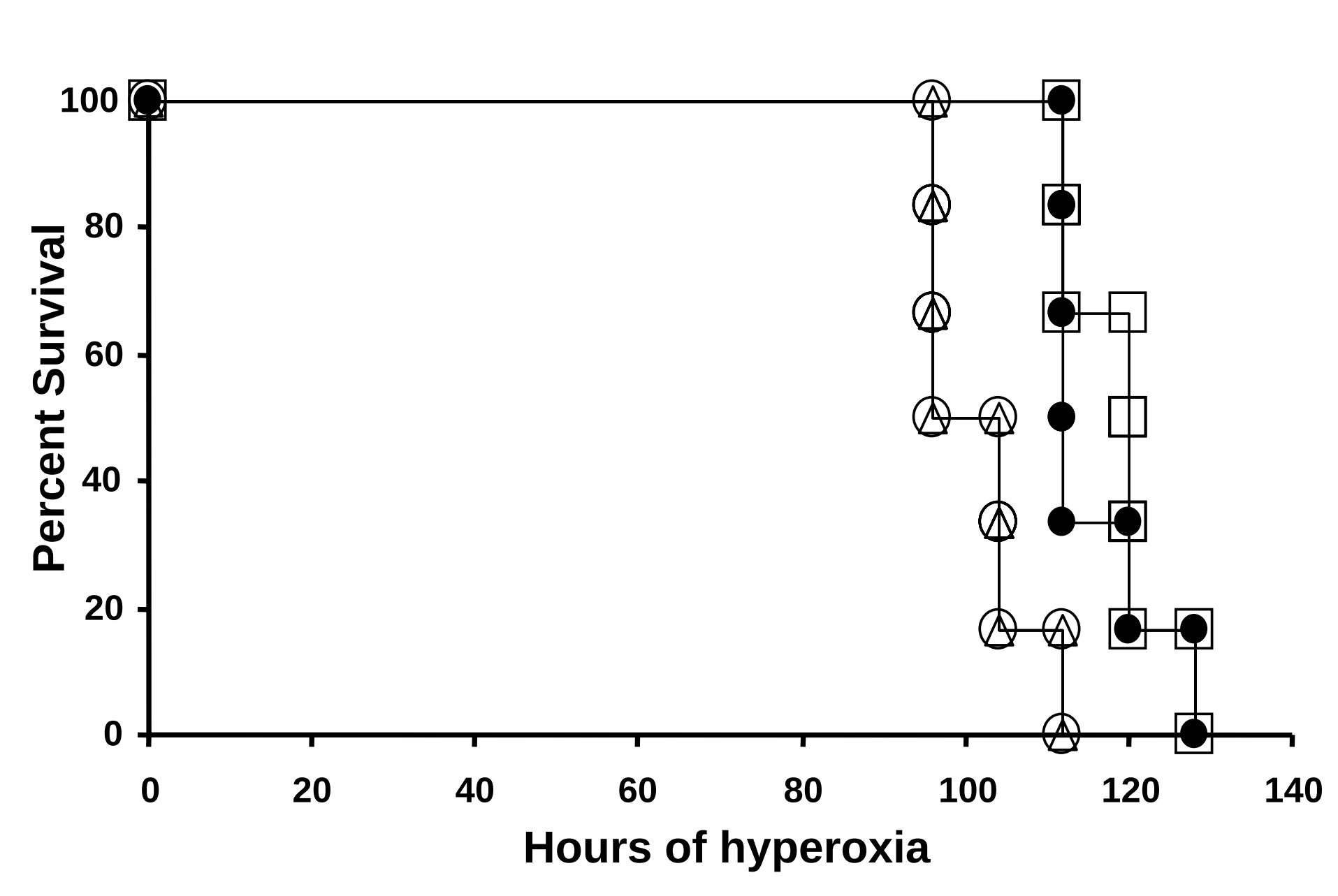
Fig. 8. Effect of the intrapulmonary administration of IL-6 siRNA on *Bach1*$^{-/-}$ mice during hyperoxic exposure. The *Bach1*$^{-/-}$ mice were intratracheally administerd either IL-6 siRNA or NS siRNA 2 h before the start of hyperoxic exposure. The IL-6 levels in BALF obtained from the mice after 72 h of hyperoxic exposure were measured and a survival analysis was performed. (A) IL-6 levels in BALF. **P* < 0.05: *Bach1*$^{-/-}$ mice administered IL-6 siRNA (solid bar) versus the *Bach1*$^{-/-}$ mice administered NS siRNA (open bar). The data are shown as the mean ± SEM of 5 animals in each group.
(B) The survival of *Bach1*$^{-/-}$ mice administered either IL-6 siRNA (open circles) or NS siRNA (open triangles) following hyperoxic exposure. Eight mice from each group

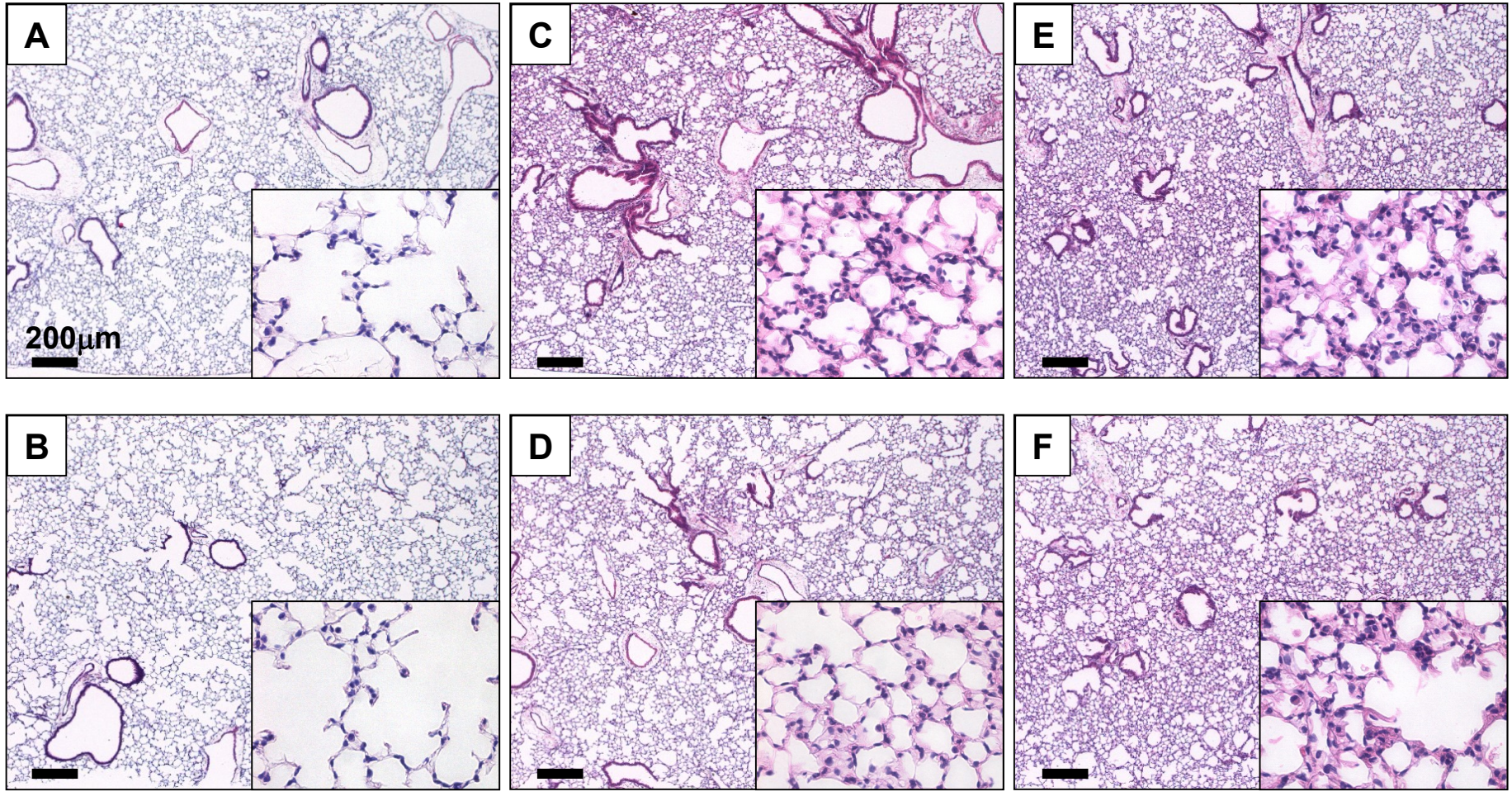
were exposed to 90% O_2 until all animals had died. P =0.029: The *Bach1*$^{-/-}$ mice administered IL-6 siRNA versus *Bach1*$^{-/-}$ mice administered NS siRNA.

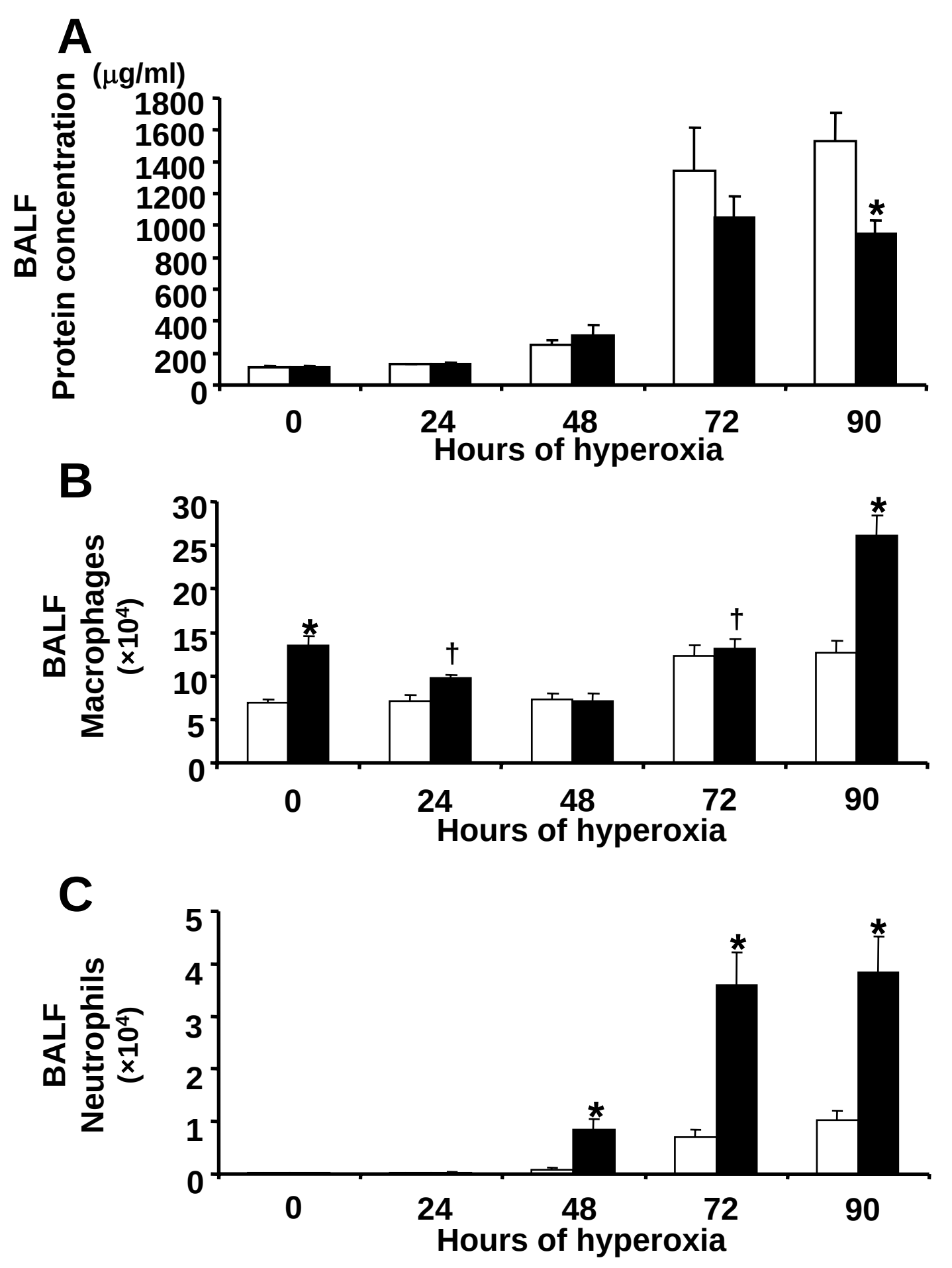
Fig. 9. A ChIP analysis using primers specific for IL-6 promoter. A ChIP analysis was performed on LLC and RAW 264 cells incubated in the presence or absence of H_2O_2 as oxidative stress. DNA immunoprecipitated with anti-Bach1 antibody and normal rabbit IgG (negative control) was analyzed by PCR using primers specific for the IL-6 promoter region.

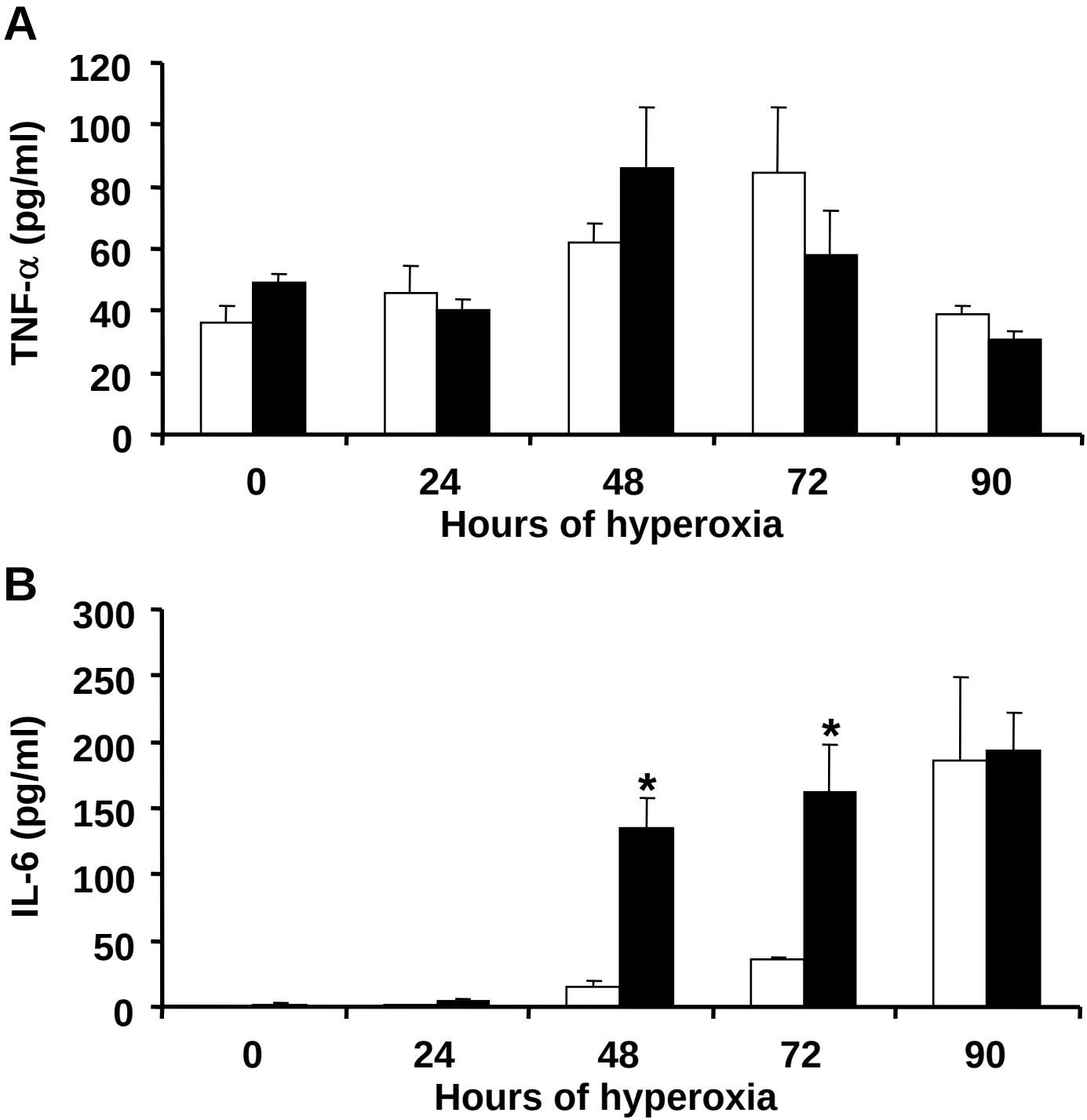
Fig. 10. A schematic figure illustrating the interactions among Bach1, IL-6, and HO-1 in hyperoxic lung injury.

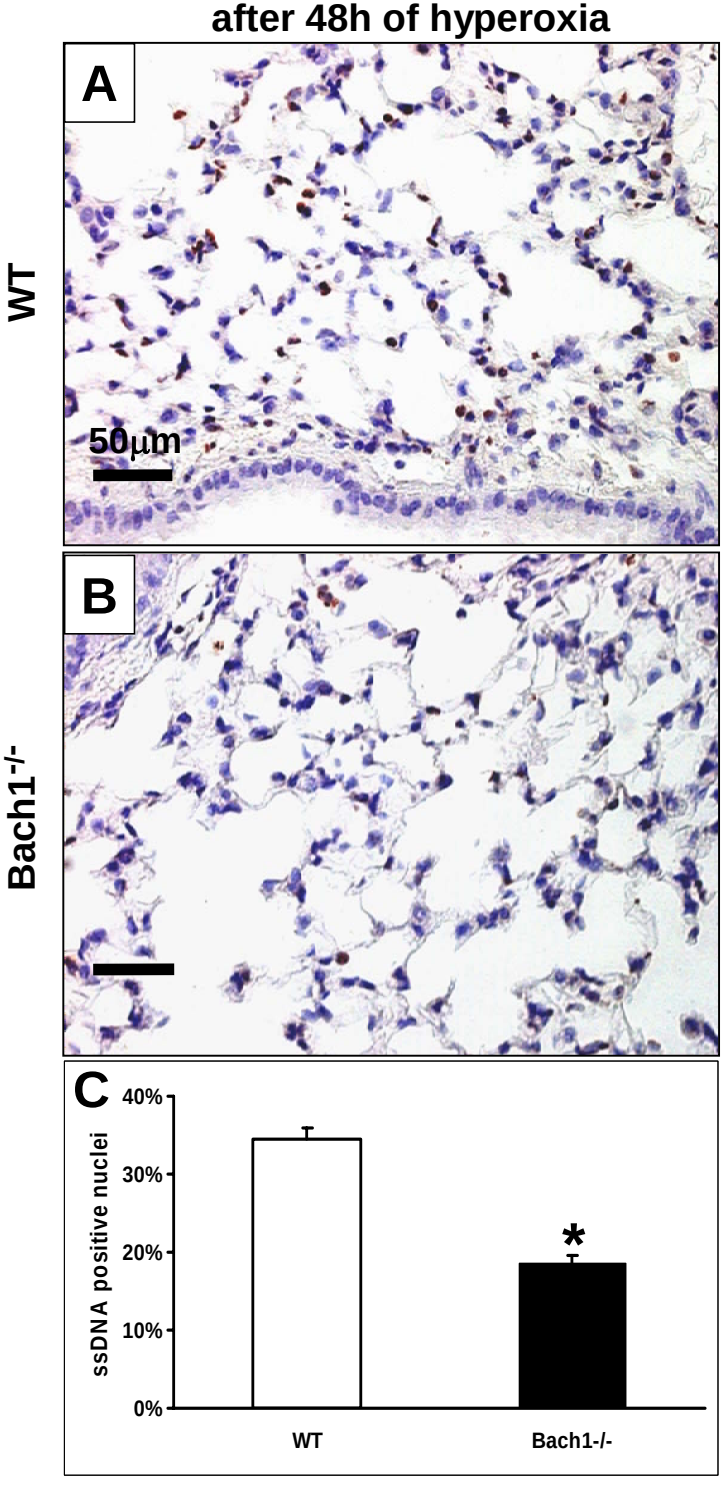

A
(ng/mg protein)
HO-1 protein level
45
40
35
30
25
20
15
10
5
0
*
*
*
*
*
0
24
48
72
90
Hours of hyperoxia
B
HO-2 expression level
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
48
90
Hours of hyperoxia

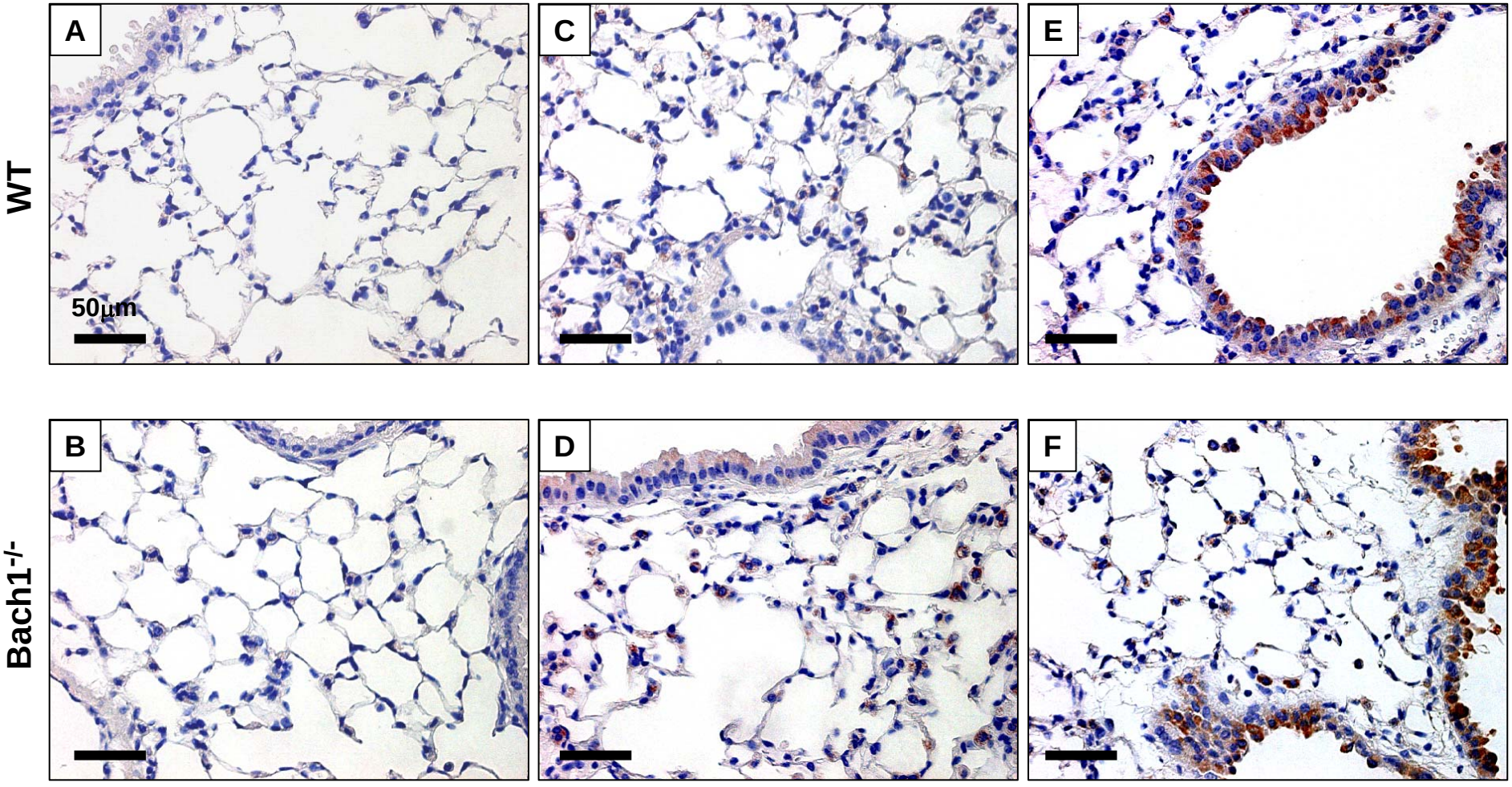

Percent Survival
100
80
60
40
20
0
0
20
40
60
80
100
120
140
Hours of hyperoxia


A
C
E
B
D
F
WT
Bach1-/-
200μm
before hyperoxia
after 48h of hyperoxia
after 90h of hyperoxia

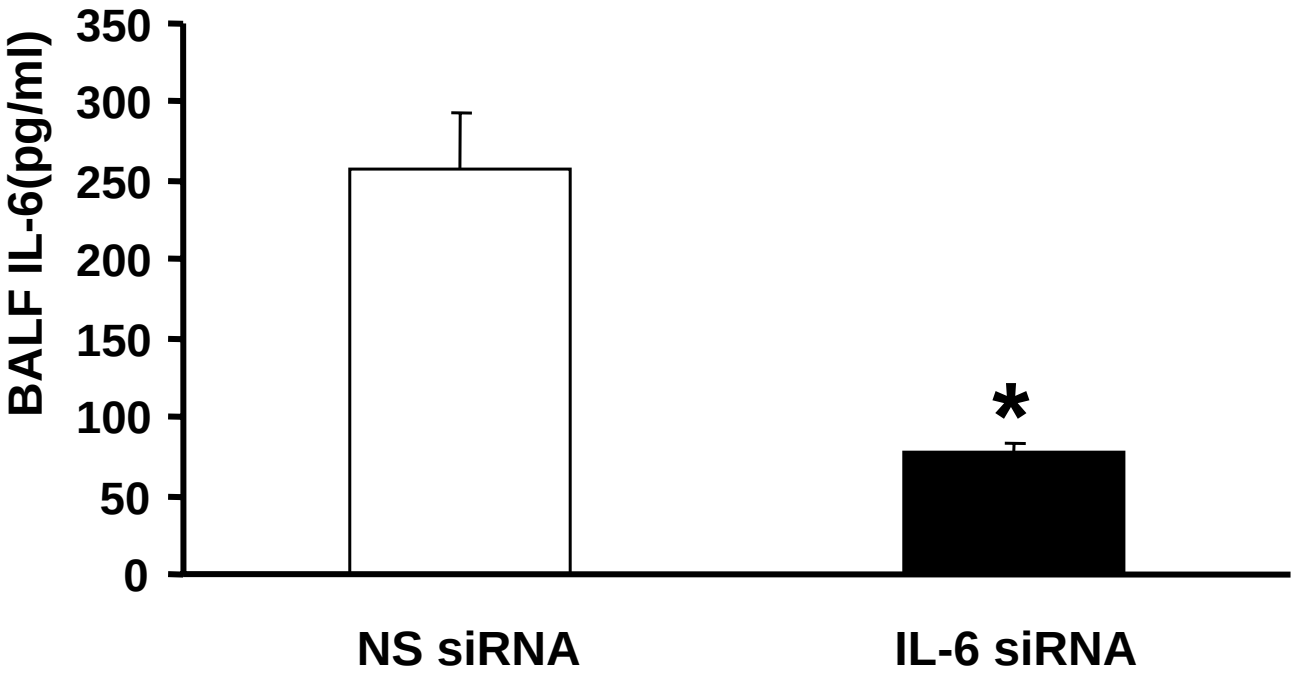

A
(μg/ml)
BALF
Protein concentration
1800
1600
1400
1200
1000
800
600
400
200
0
0
24
48
72
90
Hours of hyperoxia
*
B
BALF
Macrophages
(×10⁴)
30
25
20
15
10
5
0
*
†
†
*
0
24
48
72
90
Hours of hyperoxia
C
BALF
Neutrophils
(×10⁴)
5
4
3
2
1
0
*
*
*
0
24
48
72
90
Hours of hyperoxia


A
120
100
80
60
40
20
0
TNF-α (pg/ml)
0
24
48
72
90
Hours of hyperoxia
B
300
250
200
150
100
50
0
IL-6 (pg/ml)
*
*
0
24
48
72
90
Hours of hyperoxia

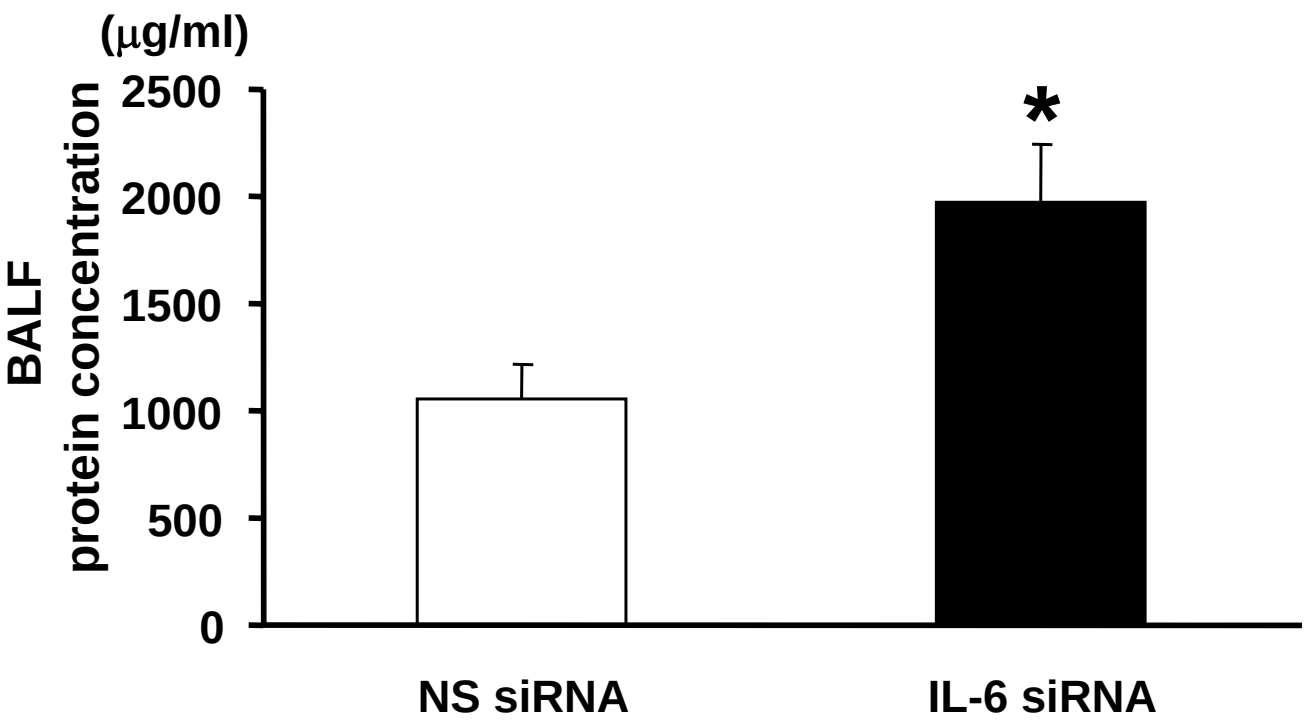

after 48h of hyperoxia
A
WT
50μm
B
Bach1-/-
C
ssDNA positive nuclei
40%
30%
20%
10%
0%
*
WT
Bach1-/-

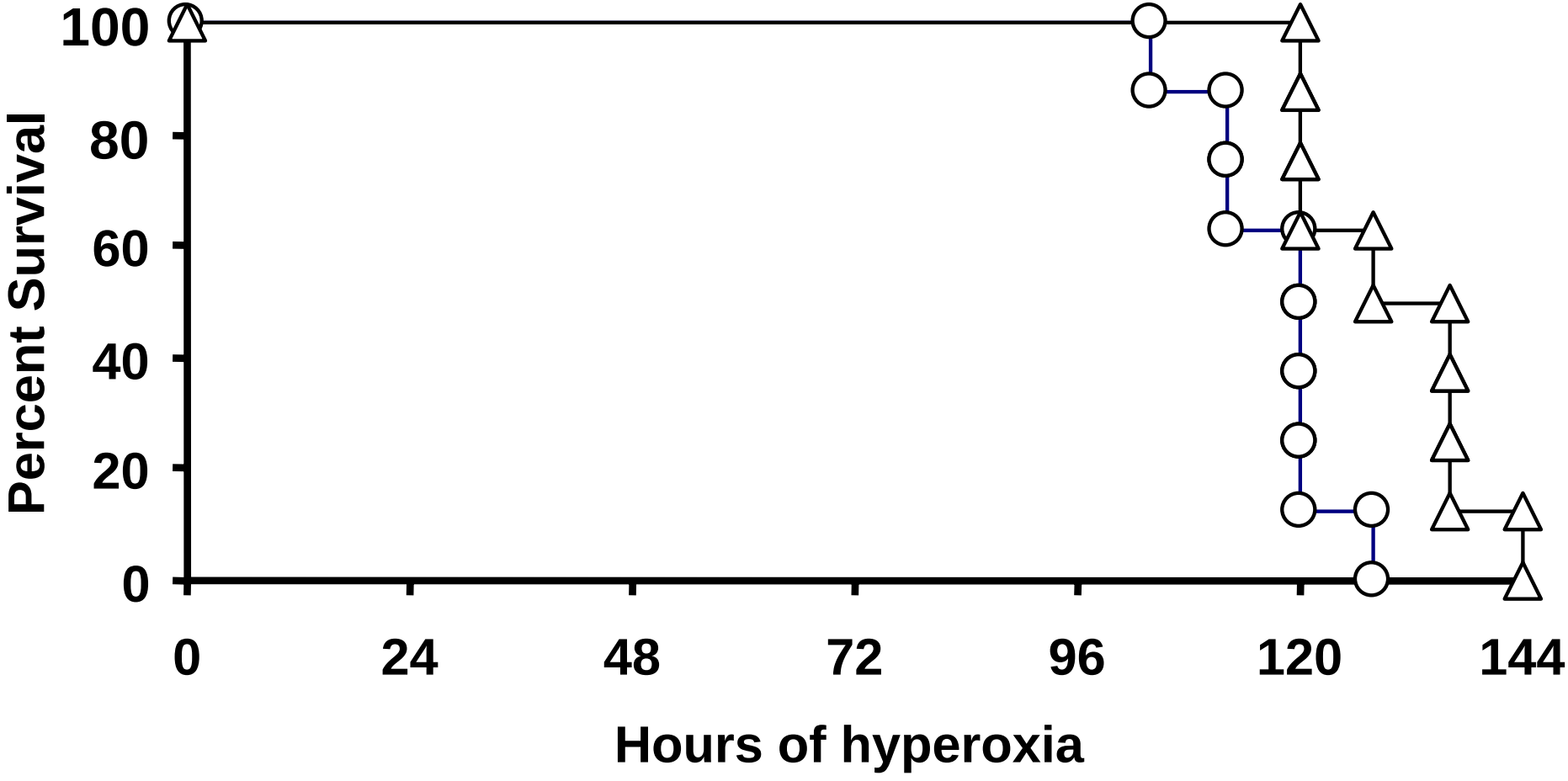

A
C
E
B
D
F
WT
Bach1-/-
50µm
before hyperoxia
after 48h of hyperoxia
after 90h of hyperoxia

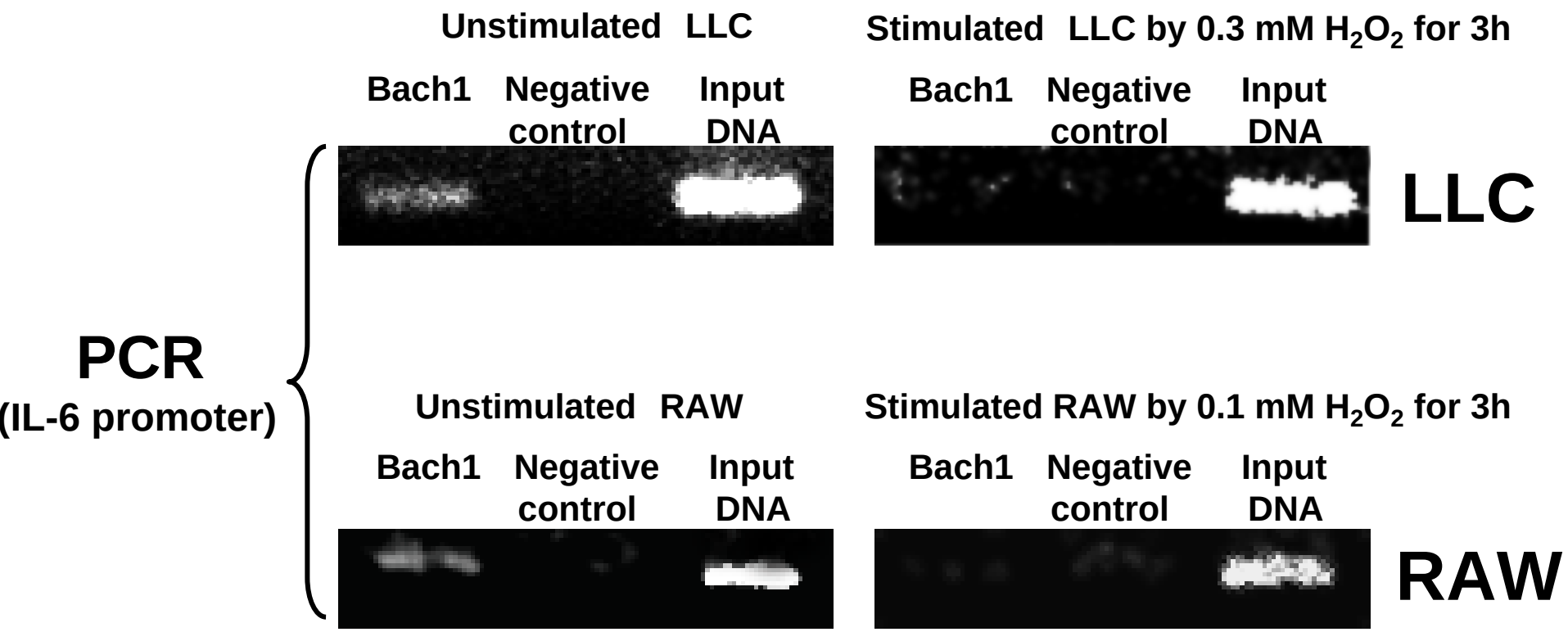
A

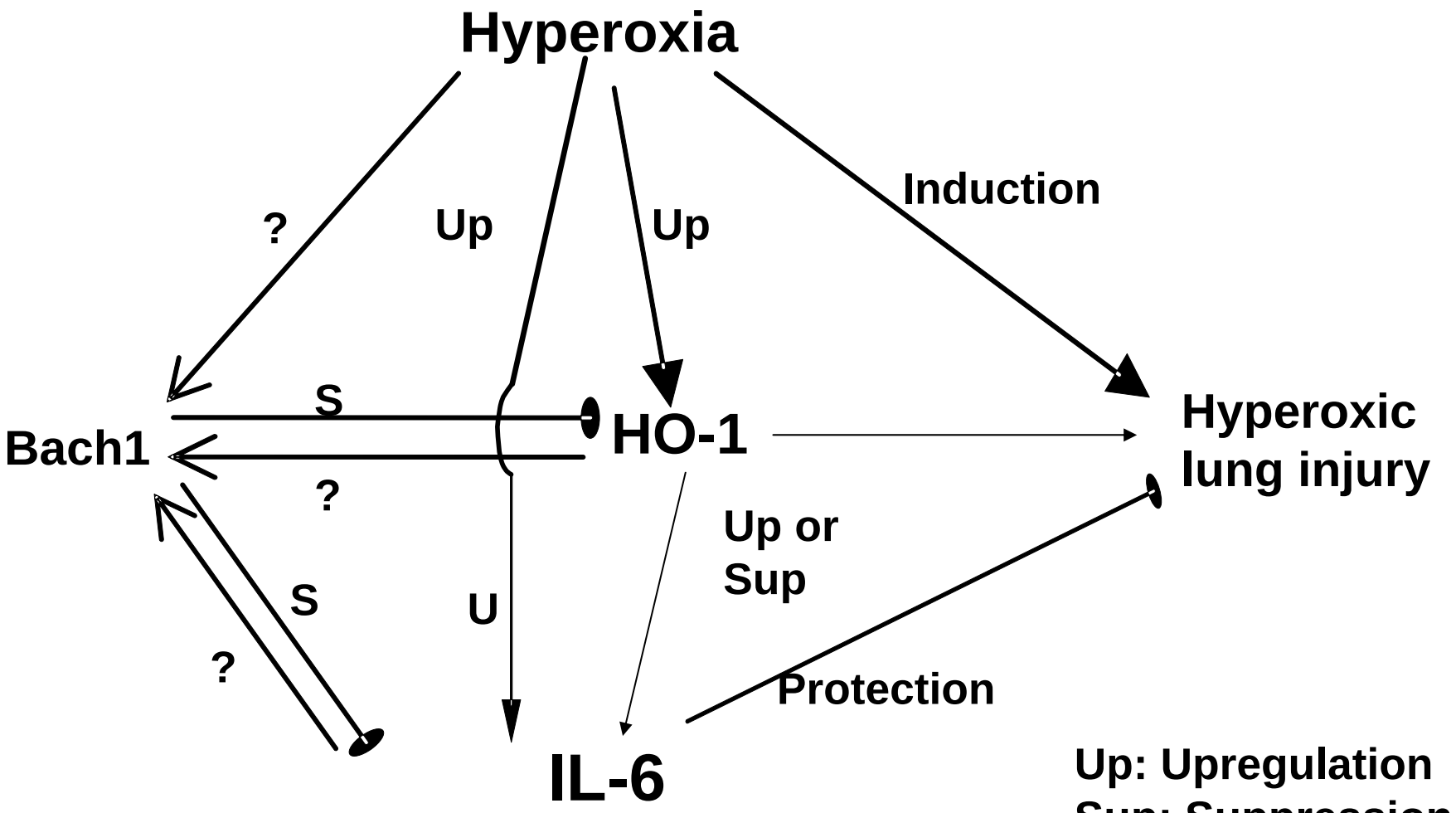

BALF IL-6(pg/ml)
350
300
250
200
150
100
50
0
*
NS siRNA
IL-6 siRNA

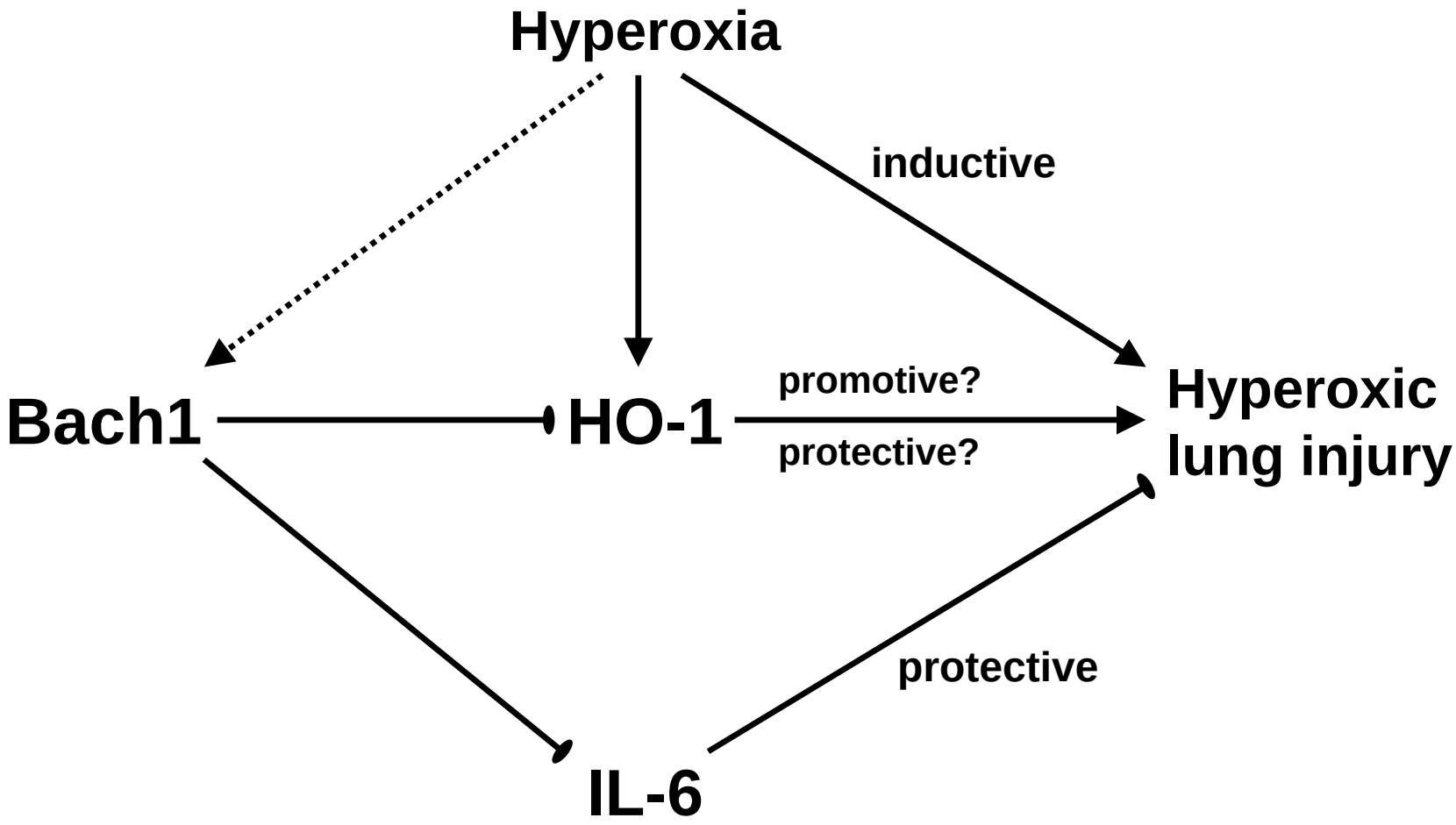
B


(μg/ml)
BALF
protein concentration
2500
2000
1500
1000
500
0
*
NS siRNA
IL-6 siRNA


Percent Survival
100
80
60
40
20
0
0
24
48
72
96
120
144
Hours of hyperoxia


Unstimulated LLC
Bach1
Negative control
Input DNA
Stimulated LLC by 0.3 mM H_2O_2 for 3h
Bach1
Negative control
Input DNA
LLC
PCR
(IL-6 promoter)
Unstimulated RAW
Bach1
Negative control
Input DNA
Stimulated RAW by 0.1 mM H_2O_2 for 3h
Bach1
Negative control
Input DNA
RAW


Hyperoxia
?
Up
Up
Induction
S
Bach1
HO-1
Hyperoxic
lung injury
?
Up or
Sup
S
U
?
Protection
IL-6
Up: Upregulation
Sup: Suppression


Hyperoxia
inductive
Bach1
HO-1
promotive?
protective?
Hyperoxic
lung injury
protective
IL-6