Evidence for a role of heat shock factor 1 in inhibition of NF-κB pathway during heat shock response-mediated lung protection

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Wirth, Delphine, Fabrice Bureau, Dorothée Melotte, Elisabeth Christians, and Pascal Gustin. Evidence for a role of heat shock factor 1 in inhibition of NF-κB pathway during heat shock response-mediated lung protection. Am J Physiol Lung Cell Mol Physiol 287: L953–L961, 2004. First published June 25, 2004; doi:10.1152/ajplung.00184.2003.—Heat shock transcription factor (HSF)-1 is recognized as a central component of the heat shock response, which protects against various harmful conditions. However, the mechanisms underlying the protection and the role of HSF-1 in these mechanisms have not yet been clearly elucidated. Using HSF-1 knockout mice (Hsf1−/−), we examined whether heat shock response-mediated lung protection involved an inhibition of the proinflammatory pathway via an interaction between HSF-1 and NF-κB, in response to cadmium insult. The HSF-1-dependent protective effect against intranasal instillation of cadmium (10 and 100 μg/mouse) was demonstrated by the higher protein content (1.2- and 1.4-fold), macrophage (1.6- and 1.9-fold), and neutrophil (2.6- and 1.8-fold) number in bronchoalveolar fluids, higher lung wet-to-dry weight ratio, and more severe lung damage evaluated by histopathology in Hsf1−/− compared with wild-type animals. These responses were associated with higher granulocyte/macrophage colony-stimulating factor (GM-CSF) (4), an increasing interest has been focused on HS preconditioning-induced inhibition of NF-κB activation in response to proinflammatory stimuli (30, 42, 47). Although HSF-1 was suspected to be involved in this HS-mediated effect, recent data obtained with Hsf1−/− embryonic fibroblasts suggested that HSF-1 was not responsible for this inhibition (29). Therefore, a better understanding of the role of HSF-1 in NF-κB pathway inhibition requires further investigations in other cell types and also in intact animals. This question was directly addressed in this work using Hsf1 knockout mice exposed to cadmium (Cd).

Cadmium provokes lung damage and inflammation (1, 3, 31) involving cytokine production (24). Interestingly, this heavy metal is also known as a strong inducer of the HS response (15, 41). The protective role of HSF-1 against Cd-induced lung injuries has been recently identified (40), but the possible interactions between HSF-1 and proinflammatory pathways were not known. In vitro studies showed that Cd activated the HSF-1 and NF-κB pathways by increasing the level of oxidative stress (8, 14, 20, 21), but both phenomena were analyzed separately.

The objective of this study was to examine in vivo whether the HSF-1-mediated protection induced by Cd against its own toxic effects involved an inhibition of TNF-α and GM-CSF, two cytokines thought to play a part in Cd-induced inflammation in the lung. Furthermore, a potential interaction between...
HSF-1 and NF-κB was investigated by measuring the level of activation of this factor in WT and HSF-1-deficient mice after intranasal instillation of Cd.

MATERIALS AND METHODS

Mice. Experiments were performed in WT and HSF-1-deficient (Hsf1−/−) female mice (body wt 20 ± 1 g, 9 wk old). WT C57BL/6 mice were purchased from Charles River Laboratories (IFFA CREDO, Bruxelles, Belgium). Hsf1−/− mice were obtained from the laboratory of Ivor Benjamin (Univ. of Texas Southwestern Medical Center, Dallas, TX) and have been previously described (45). A breeding colony of Hsf1−/− knockout mice with an enriched C57BL/6 background was developed in our animal facility (Faculty of Veterinary Medicine, Univ. of Liege, Belgium) by mating Hsf1+/− males with WT C57BL/6 females for five or six generations. Then, Hsf1+/− males and females were intercrossed to produce Hsf1−/− mice with >99% of C57BL/6 background. Mice were housed in a temperature- and humidity-controlled animal house, maintained at 21°C with a 12-h light-dark cycle. A balanced diet and water were supplied ad libitum. The ethics committee of the University of Liege approved the study.

Cd treatments. Cd was administered by intranasal instillation to WT or Hsf1−/− mice following anesthesia by intraperitoneal injection of a mixture of medetomidine (Orion, Finland) and ketamine (Merial, France) (0.6 μg/g and 0.04 mg/g body wt, respectively). For intranasal instillation, a Gilson pipette was used to dispense 20 μl of saline solution containing 0 (control), 10, or 100 μg of CdCl2 in the nasal cavities.

Bronchoalveolar lavage procedure and analyses. Mice were killed by cervical dislocation. Lungs were washed with 1 ml of PBS (GIBCO, Paisley, UK) through a tracheal cannula, and ~0.7 ml of bronchoalveolar lavage (BAL) was collected. BAL was centrifuged (5,000 g for 2 min) and the supernatant fluid phase was aliquoted and stored at −80°C until cytokine and protein concentrations were assessed. Pelleted cells were resuspended in 200 μl of PBS. Total cells were counted with a hemocytometer, and cell differentials were determined in 200 μl of BAL fluid on Cytospin slides stained with May-Grunwald and Giemsa stain (Merck, Darmstadt, Germany). The protein concentration in cell-free BAL was determined by bicinchoninic acid protein assay (Pierce, PerbioScience, Erembodegem, Belgium) with bovine serum albumin as a standard.

Cytokine assays. The concentrations of TNF-α and GM-CSF were quantified in the BAL fluid supernatants by ELISA using mouse-specific kits (BioSource International, Nivelles, Belgium) according to the manufacturer’s instructions. In each reaction well, 100 μl of sample were added to reagents without dilution.

Nuclear and cytoplasmic protein extraction, EMSAs, and Western blot analysis. Nuclear proteins were extracted from left washed lungs as previously described (6). Briefly, sample was homogenized in 5 ml of cytoplasmic buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 2 mM MgCl2, 0.1 mM EDTA, 0.2% Nonidet P-40, and 1.6 mg/ml protease inhibitors (Complete; Boehringer Mannheim, Mannheim, Germany) and supplemented with 3 mM of the protease inhibitor disopropyl fluorophosphates (Sigma, Bornem, Belgium). Extracts were centrifuged (1,500 g, 5 min at 4°C). Pelleted nuclei were washed and resuspended in nuclear buffer (20 mM HEPES, pH 7.9, 1.5 mM MgCl2, 0.2 mM EDTA, 0.63 M NaCl, 25% glycerol, 1.6 mg/ml protease inhibitors, 3 mM disopropyl fluorophosphates), incubated for 20 min at 4°C, and centrifuged for 15 min at 11,000 g. Protein was quantified in supernatants with the Micro BCA protein assay reagent kit (Pierce, Rockford, IL). A 32P-labeled oligonucleotide containing the consensual κB sequence (6) or the consensual heat shock element sequence (5′-CTAGAAGCTTCTAGAAGCTTCTAG-3′) was used as the probe in EMSAs. The amount of specific complexes was determined by photodensitometry of the autoradiography. Super-shift experiments were also performed as previously described (6) using rabbit antibodies recognizing the specific NF-κB subunits p50, p52, p65, RelB, and c-Rel. The antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Fig. 1. Total cell, macrophage, and neutrophil counts in bronchoalveolar lavage (BAL) fluids of wild-type (WT) and heat shock transcription factor (HSF)-1 deficient mice exposed to 10 (A) or 100 (B) μg of cadmium (Cd). Mice received an intranasal instillation of Cd or saline and were killed for BAL 4, 10, or 24 h after administration. Alveolar total cells, macrophages, and neutrophils were quantified. No significant changes were detected in other cell counts. Because no changes were recorded during 24 h in saline-treated mice, all values were pooled and presented as controls values. Results are shown as means ± SD (n = 5–6/time point). §P < 0.05 for comparison with controls in the same group. *P < 0.05 for comparison with WT at the same time point. Significant difference was recorded between the 2 doses in the same type of animals (P < 0.05, ANOVA). C, control.
Cytoplasmic protein extracts (10 μg) were added to a loading buffer [10 mM Tris-HCl, pH 6.8, 1% (wt/vol) SDS, 25% (vol/vol) glycerol, 0.1 mM 2-mercaptoethanol, and 0.03% (wt/vol) bromophenol blue], boiled, and run on a 10% SDS-PAGE gel. After electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes (Roche, Mannheim, Germany) and blocking overnight at 4°C with 20 mM Tris (pH 7.5), 500 mM NaCl, 0.2 (vol/vol) Tween 20 (Tris·HCl/Tween) and 5% (wt/vol) dry milk, the membranes were incubated for 1 h with a rabbit polyclonal recognizing mouse IκB-α (1:1,000 dilution), washed, and incubated for 45 min with peroxidase-conjugated goat anti-rabbit IgG (1:2,000 dilution; Kirkegaard & Perry, Gaithersburg, MD). The results of the reaction were revealed with the enhanced chemiluminescence detection method (ECL kit; Amersham Pharmacia Biotech, Aylesbury, UK).

Lung histology. The washed lungs were dissected and infused with 4% buffered formaldehyde at 25 cmH₂O pressure via a tracheal cannula until all pulmonary lobes were fully inflated. After overnight fixation in the same fixative at 4°C, the lung tissue was embedded in paraffin using conventional methods. Five-micrometer tissue sections were stained with hematoxylin-eosin. The degree of microscopic lung injury was analyzed using a semiquantitative system. Three sites on each slide were evaluated for several variables (congestion, septal thickening, edema, hemorrhage, and neutrophil infiltration) and given relative scores. For each component, the extent of injury was assigned a score of 0 (no injury), 1 (injury to 25% of the field), 2 (25–50%), or 3 (>50%), and the severity was graded as 0, 1, 2, or 3. A mean score for each variable for each animal was expressed as the sum of extent and severity.

Lung wet-to-dry weight ratio. The wet weight of whole lungs was measured on an electronic scale immediately after death. The lungs were then desiccated in an oven at 65°C for 72 h to determine the dry weight.

Statistical analysis. Data are expressed means ± SD and were analyzed using Statistica for Windows software (StatSoft, Tulsa, OK). Data were normally distributed (Levene median test). The effects of Cd dose, genotype, and time after instillation were assessed by ANOVA with three factors. Statistical comparisons between and within groups were made by post hoc Tukey’s test. Levels of significance were set at P < 0.05.

RESULTS

Cd-induced lung injury in WT and Hsf1−/− mice. To define the HSF-1-mediated protection in Cd-induced lung injury, WT and Hsf1−/− mice were exposed to 10 or 100 μg of Cd by intranasal instillation. Number of total cells, macrophages, and neutrophils, as well as protein concentration in BAL fluids, lung wet-to-dry weight ratio, and histopathology were evaluated 4, 10, and 24 h after the Cd instillation as indexes of the pathophysiological response. No changes were recorded up to 24 h after saline instillation, and there were no significant differences between the values recorded in WT and Hsf1−/− control mice (Figs. 1, 2, and 3). Cells in BAL fluids counted in control mice and before instillation were predominantly macrophages.

Compared with saline administration, both Cd doses caused progressive increase in the number of total cells, macrophages, and neutrophils in BAL fluids sampled from WT mice (Fig. 1). The first significant change in the total cell count was observed 24 h after instillation of 10 μg and 10 h after 100 μg of Cd. The changes occurring in mice exposed to the low and high Cd doses were partly due to an increase in the macrophage number (4.2- and 10.6-fold), but they were mainly explained by a marked rise in the neutrophil count (53- and 62-fold, respectively). In Hsf1−/− animals, Cd also provoked a time- and dose-dependent increase in cell counts compared with their respective controls. However, compared with WT mice, the total cell, macrophage, and neutrophil numbers were significantly higher in these mice 24 h after the two Cd doses.

Lung wet-to-dry weight ratio and BAL fluid protein were significantly increased by Cd instillation in WT mice (Fig 2). Lung wet-to-dry weight ratio reached high levels 10 h after 10 μg and 4 h after 100 μg, indicating significant edema, congestion, and/or hemorrhage. Proteins in BAL fluids reached maximal concentrations 10 h after 10 and 100 μg, confirming lung edema. The responses recorded in Cd-exposed Hsf1−/− mice followed the same kinetics, but the magnitude of the maximal changes was higher in these mice than in WT mice.

Tissue congestion and hemorrhage were observed macroscopically in some portions of the Cd-treated lungs compared with controls. Figure 3 shows light photographs of representative lung tissue from WT and Hsf1−/− mice exposed to either saline or Cd (10 or 100 μg). In saline-treated WT and Hsf1−/− mice, lungs appeared normal, with intact parenchyma and without inflammatory injury. Twenty-four hours after 10 μg of Cd instillation, the WT mice exhibited marked pathological changes in the peribronchiolar regions and throughout the alveoli. Inflammation was evident and characterized by neutrophil and macrophage infiltration into interalveolar septa and
peribronchiolar tissues as well as interstitial thickening and congestion. Some alveoli also seemed filled with sloughed material, perhaps due to edema and cytolysis. This lung damage was most severe after the high dose of Cd and was significantly more prominent in Hsf1−/− mice as shown by histological quantification.

**TNF-α and GM-CSF expression in WT and Hsf1−/− mice after Cd exposure.** To examine whether the HSF-1-mediated protection described above in WT mice, when compared with Hsf1−/− mice, was associated with an alteration of cytokine expression, TNF-α and GM-CSF concentrations were determined in BAL fluids from WT and Hsf1−/− mice 4, 10, and 24 h after Cd instillation. The BAL fluids of mice constitutively contained both TNF-α and GM-CSF, and there were no changes up to 24 h after saline treatment. No differences were detected between TNF-α and GM-CSF concentrations in WT and Hsf1−/− control mice (Fig. 4).

Cd increased the TNF-α concentration in a dose-dependent manner in the BAL fluids of WT animals (Fig. 4A). Maximal increase in TNF-α was recorded 24 h after both 10 and 100 μg of Cd, as described above regarding the inflammatory cell influx in BAL fluids. The TNF-α concentrations in BAL fluids were not significantly different between WT and Hsf1−/− mice, whatever the Cd dose.

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<thead>
<tr>
<th>Variable</th>
<th>WT</th>
<th>Hsf1−/−</th>
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<tr>
<td>Congestion</td>
<td>2.5 ± 0.2</td>
<td>4.0 ± 0.3</td>
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<tr>
<td>Septal thickening</td>
<td>3.0 ± 0.4</td>
<td>4.5 ± 0.6</td>
</tr>
<tr>
<td>Edema</td>
<td>1.5 ± 0.3</td>
<td>2.0 ± 0.4</td>
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<tr>
<td>Hemorrhage</td>
<td>2.0 ± 0.5</td>
<td>3.0 ± 0.6</td>
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<tr>
<td>Neutrophil infiltration</td>
<td>3.5 ± 0.7</td>
<td>5.0 ± 0.9</td>
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*P < 0.05 for comparison with WT mice after the same Cd dose. In all Cd-treated mice, significant difference with saline-treated mice was recorded.

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**Fig. 3.** Light micrographs of stained sections of washed lung from WT and HSF-1-deficient mice 24 h after intranasal instillation of Cd or saline. A: normal lung of WT mice. B: normal lung of HSF-1-deficient mice. C: WT mice after instillation of 10 μg of Cd showing generalized alveolar lesions (arrow), congestion (blue arrowheads), cell infiltration in interalveolar septa and interstitium (black arrowheads), and septal thickening. D: HSF-1-deficient mice after instillation of 10 μg of Cd showing similar lesions to WT mice, but more severe cell infiltration into peribronchiolar tissue (arrowhead). E: WT mice after instillation of 100 μg of Cd showing edema (arrow) and cell infiltration. F: HSF-1-deficient mice after instillation of 100 μg of Cd showing more severe alveolar lesions (arrow) and cell infiltration (arrowheads). Magnification is ×250 for lung. Histogram shows degree of microscopic injury scored for each indicated variable. Histological results are presented as means ± SD (n = 4/group). *P < 0.05 for comparison with WT mice after the same Cd dose. In all Cd-treated mice, significant difference with saline-treated mice was recorded.
Nuclear extracts from all Cd-treated animals than in the former, 4 and 24 h after Cd instillation, as the level of activity was significantly higher in the latter animals compared with WT mice, this difference was clearly less marked for the top bands.

To characterize the NF-κB complexes, supershift experiments using antibodies directed against the various members of the NF-κB family (i.e., p50, p52, p65, RelB, and cREL) were performed (Fig. 5B). Anti-p50 antibodies supershifted the faster migrating complex (bottom band) and the slower migrating complex (top band), which was also supershifted by anti-p65 antibodies. This identified the two specific Cd-activated NF-κB complexes as the p50 homodimer and the p65/p50 heterodimer. These results, together with the analysis of NF-κB activity, suggested that the binding activity of the p65/p50 heterodimer, and particularly of the p50 homodimer, was higher in HSF-1-deficient mice compared with WT mice (Fig. 5, A and B).

Having demonstrated that HSF-1 inhibited Cd-mediated NF-κB activation, we therefore wanted to determine the effect of HSF-1 on inhibitory κB (iκB) stability after Cd instillation. Treatment with 10 and 100 μg of Cd caused rapid degradation of iκB in both the WT and Hsf1−/− mice compared with controls (Fig 5C). The Cd-induced iκB degradation was more marked in Hsf1−/− animals compared with WT mice 24 h after instillation.

HSF-1 activation in WT mice after Cd exposure. Results based on the use of Hsf1−/− mice indicated a role of HSF-1 in lung protection against Cd involving inhibition of proinflammatory processes. To attribute and confirm the functional role for HSF-1 in our model, activation of this factor by Cd and kinetics of its activation was investigated by measuring the HSF-1 binding activity in the lungs of WT mice 1, 2, 3, 4, 10, and 24 h after Cd instillation. As shown in Fig. 6, Cd increased DNA-binding activity of HSF-1 relative to that seen in corresponding controls. This increase reached maximal magnitude 2 h after Cd exposure, and after that the induction disappeared. Therefore, HSF-1 activation preceded NF-κB inhibition.

In contrast, instillation of Cd was followed by a peak of GM-CSF concentration 10 h after both 10 and 100 μg, indicating that GM-CSF release preceded macrophage and neutrophil recruitment. In addition, the GM-CSF concentrations were significantly higher in Hsf1−/− animals compared with WT mice.

NF-κB activation and inhibitory κB degradation in WT and Hsf1−/− mice after Cd exposure. Because GM-CSF expression is modulated by HSF-1 and since NF-κB is a major regulator of proinflammatory processes (4), the effect of HSF-1 on NF-κB activation was investigated by measuring the NF-κB DNA-binding activity in the lungs of WT and Hsf1−/− mice 4 and 24 h after Cd instillation.

The lungs of saline-treated WT mice exhibited weak basal NF-κB binding activity (Fig. 5A). Cd induced marked NF-κB activation as early as 4 h after instillation of both doses in these mice, indicating that NF-κB activation in lung tissue preceded cytokine release in BAL fluids. In addition, binding activity level remained elevated at 24 h. Whereas the basal NF-κB binding activity was not different in the WT and Hsf1−/− mice, the level of activity was significantly higher in the latter animals than in the former, 4 and 24 h after Cd instillation, as shown by densitometry. Nuclear extracts from all Cd-treated mice exhibited activation of distinct NF-κB complexes as shown by the two bands detected in each sample. Whereas the bottom band was systematically more intense in Hsf1−/− than in WT mice, this difference was clearly less marked for the top bands.

Discussion

The inhibition of inflammatory response to various insults by the HS response involves different pathways protecting cells, organs, or organisms. Although the involved mechanisms are not yet clearly elucidated, Hsps and their major regulatory partner HSF-1 are recognized as playing an important role in this protection. However, although prior induction of HS response could modulate the activation of NF-κB and the production of cytokines in response to a proinflammatory stimulus (42, 43, 47), the involvement of HSF-1 in this effect has remained a matter of debate (29, 45). The use of HSF-1-deficient mice enabled us to unambiguously identify the role of this factor in the HS response effects on the lung damage, NF-κB activation, and cytokine release, after Cd exposure.

Our results confirmed that acute Cd exposure elicited HSF-1-dependent protective mechanisms against its own toxic effects, as recently characterized in mice (40). Indeed, compared with WT mice, Hsf1−/− mice exhibited higher lung wet-to-dry weight ratios after instillation of 10 μg of Cd and more a marked increase in protein concentrations and inflam-
matory cell numbers in BAL fluids, confirming the more severe histopathological signs of lung damage detected after 10 and 100 µg. The investigation of the cytokine concentrations in BAL fluids indicated that Cd stimulated both TNF-α and GM-CSF pulmonary expression in mice. The increase in the TNF-α concentration in BAL fluids, occurring simultaneously with the increase in macrophage and neutrophil numbers, might be interpreted as a consequence rather than a mediator of the inflammatory cell influx. This was in accordance with previous data showing that TNF-α was not responsible for the hepatotoxicity in mice exposed to Cd (79). In contrast, GM-CSF increase, preceding maximal macrophage and neutrophil

Fig. 5. Representative analysis of NF-κB activation (A), specific active NF-κB complexes (B), and inhibitory κB (IκB) degradation (C) in the lungs of WT and HSF-1-deficient mice exposed to Cd. Mice received an intranasal instillation of Cd or saline and were killed 4 or 24 h after administration. Nuclear and cytoplasmic extracts were prepared from lung tissue and were analyzed for NF-κB binding activity by EMSA (A) and IκB-α stability by Western blot (C), respectively. The line of mice, the dose of Cd administered, and the time at which animals were killed are indicated above each lane. The open and closed arrows (A) indicate specific slower and faster migrating NF-κB complexes. Histograms represent the mean (± SD) densitometry values of 4 individual experiments. §P < 0.05 for comparison with controls in the same group. *P < 0.05 for comparison with WT at the same time point. B: characterization of specific active NF-κB complexes was performed by supershift analyses conducted with specific antibodies as indicated. The specific complexes are indicated by arrows. Supershifts of p50 and p65 are indicated by closed and open arrowheads, respectively. Ab, antibodies.
Because all these changes occurred after the increase in lung of cytokine expression, in agreement with Xiao et al. (45).

This is the first in vivo evidence of an effect of HSF-1 on NF-κB activity (10, 26, 47), but evidence for the role of HSF-1 in this effect is still lacking. Finally, GM-CSF can regulate NF-κB activity. HSF-1-mediated inhibition of GM-CSF expression could then reinforce inhibition of NF-κB activation. Together, our results suggest that, during the early Cd-induced lung injuries, an interaction between HSF-1 and NF-κB seems to be involved in the protective effect of the HS response. Afterward, at 24 h after Cd instillation, different mechanisms could contribute to inhibition of the NF-κB pathway, but HSF-1 still appears as a major mediator of these.

As mentioned above, the comparison of cytokine production in WT and Hsf1−/− mice revealed that HSF-1 moderated GM-CSF expression in response to Cd. In contrast, the lack of influence of HSF-1 on TNF-α raises a question about the role played by NF-κB in the control of these cytokines, since its activity was higher in Hsf1−/− mice. A possible explanation could be given by the differential effect of the p65/p50 heterodimer and the p50 homodimer on cytokine expression, according to their respective activity. Whereas the levels of binding activity of both complexes were higher in Hsf1−/− mice compared with WT animals, the difference was especially marked regarding p50. This complex is suspected to have a specific repressor effect on the TNF-α gene but not on the GM-CSF gene (22), although p65/p50 is activator of both cytokines’ expression (4, 17). Therefore, the Cd-induced TNF-α expression depends on the combination of opposite actions of both p65/p50 and p50. In Hsf1−/− mice, the repressive effect of the high level of p50 activity could abrogate the positive effect of p65/p50 on TNF-α. In contrast, the control of GM-CSF is less complex, and the higher GM-CSF concentrations recorded in Hsf1−/− mice could result from the positive control exerted by p65/p50, whereas p50 did not influence this control. A verification of this hypothesis will require further experiments.

In summary, several pathways appear to contribute to HS response-mediated lung protection against chemical-induced lung injury, including cytotoxic functions of Hsps under the control of HSF-1 and interaction between the HS response and the proinflammatory process (30, 44). In the Cd-induced lung injury model, the inhibition of inflammatory responses by the HS response involves a decrease of cytokine expression, particularly of GM-CSF, which was related to an inhibition of NF-κB activation. This is the first in vivo demonstration that HSF-1 is a major mediator of this latter effect. Given the fundamental roles of the HS response and the NF-κB pathway in cellular function, it is probable that the interaction between HSF-1 and NF-κB has the potential to impact on the course and outcomes of critically ill patients.

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HSF-1 AND NF-κB IN LUNG PROTECTION

GRANTS

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