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Differential heat shock gene *hsp70-1* response to toxicants revealed by in vivo study of lungs in transgenic mice

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Abstract: Members of heat shock proteins (Hsp70) family have been considered to respond to a large variety of stressful conditions. But it was suggested that, in pulmonary cells, Hsp response depends more closely on the type of stimulus. The lungs are critical organs potentially subjected to air pollution affecting respiratory function and, therefore, these organs are of particular interest with regard to the stress response. To investigate the stress dependence of Hsp70 response in lungs, we created transgenic mice where the firefly luciferase reporter gene is under the control of the murine *hsp70-1* promoter and exposed them to different sublethal toxic conditions. For each condition, the level of transgene induction and pulmonary toxicity were assessed. We found that *hsp70-1* promoter was stimulated by heat shock and cadmium but not by ozone, paraquat, and parathion, even if these chemicals induced respiratory distress and lung inflammation. Similar observations were made when expression of the endogenous *hsp70-1* gene was analyzed, indicating that our transgenic model was accurately detecting *hsp70-1* induction. Thereby, it appeared that *hsp70-1* response is selective and depends on signaling pathways triggered by the toxicants rather than by their pathologic toxicity per se. Furthermore, because all the chemicals used in our study have been previously described to increase the level of oxidative stress, it indicates that there is no direct and simple correlation between *hsp70-1* response and the level of oxidative stress, but more specific oxidative patterns should be involved in Hsp regulation.

INTRODUCTION

Expression of the inducible 70-kDa heat shock protein (Hsp70) is activated by a large variety of stressful conditions (Ryan and Hightower 1996; Kiang and Tsokos 1998), so that *hsp70* induction is often considered as a nonspecific response. But based on comparison of several in vitro studies, Wong and Wispe (1997) suggested that at least in pulmonary cells, the pattern of stress protein expression might depend more closely on the type of stimulus. Pulmonary tissue is particular with regard to the stress response because it represents the main target for environmental aggressions and expresses a higher basal level of inducible Hsp70 than do other organs (Blake et al 1990; Tanguay et al 1993). Nevertheless, this stimulus-dependent induction of *hsp* genes in the lungs still needs to be further demonstrated in vivo, using the same biological model and identical experimental conditions.

Few laboratories have reported their results on a systematic analysis of Hsp expression using a large variety of toxicants (Fischbach et al 1993; Sacco et al 1997; Steiner et al 1998; Ait-Aissa et al 2000), and other studies using the same toxicant, for example ozone (O_3), gave contradictory results (Su and Gordon 1997; Wu et al 1999). The most relevant to lung tissue was the study by Cohen et al (1991), showing that surface acidification, heat, and arsenite caused the synthesis of Hsp72 in respiratory cells but O_3 and hydrogen peroxide did not. Therefore, to reveal the physiologic significance of these data, it is important to evaluate the effects of these toxicants on intact organisms.

The objectives of our study were (1) to design a bio-assay for an easy evaluation of *hsp70* gene induction in organs of an animal model and then, using our in vivo model, and (2) to investigate the potential stress dependence of the Hsp70 response in the lungs after exposure to several sublethal toxic conditions. Our in vivo model is transgenic mice, which have been created with a hybrid gene, coupling *hsp70-1* promoter to the firefly luciferase reporter gene (HSP70.1Luc transgene). Taking advantage of the luciferase assay, this model allows a fast and easy evaluation of promoter activation.

Atmospheric pollutants, pesticides, and heavy metals are well known to be widespread pollutants, and their effects on respiratory system are relatively well studied. In this study, we have selected 4 different pollutants (O_3, paraquat, parathion, and cadmium) and analyzed their effects on *hsp70* expression. O_3 is a major component of

photochemical oxidant air pollution. The mechanisms of O_3 toxicity involve the formation of oxygen-free radicals responsible for membrane lipid oxidation (Pryor et al 1995). Paraquat, a potent herbicide, causes an acute damaging phase in the lung. A specific transport process explains its toxicity in pneumocytes where paraquat elevates intracellular levels of superoxide through redox cycle (Smith 1987). Parathion is an organophosphate insecticide with a weak cholinesterase inhibitor activity; it is responsible for lung injury through an unknown mechanism, though some have described that such pesticides generate reactive oxygen species (Bagchi et al 1995). Cadmium, a transition metal and a well-known inducer of Hsp70 synthesis (Zou et al 1998), exhibits cytotoxic and functional effects on respiratory tract (Ueda et al 1989; Bajpai et al 1999). Cadmium exposure leads to the accumulation of altered proteins and increases the level of oxidative stress by mechanisms reducing the glutathione (GSH) pool (Stohs and Bagchi 1995).

In this article, we have tested the hypothesis of stress dependence of Hsp70 expression by analyzing the effects of 4 different stressors (O_3, paraquat, parathion, and cadmium) on the induction of *hsp70-1* in the lungs as well as their pulmonary toxicity.

MATERIALS AND METHODS

Chemicals

$CdCl_2$ was purchased from Baker (Deventer, Netherlands), methyl paraquat was from Sigma (St Louis, MO, USA), and parathion-ethyl was from Riedel-de Haën (Hannover, Germany).

Transgenic mice generation

Transgenic lines were derived from the C57BL/6J × CBA mice by the classical method of pronucleus microinjection (Hogan et al 1986). The injected construct was the linearized HSP70.1Luc plasmid deoxyribonucleic acid (DNA) containing the complementary DNA (cDNA) of the firefly luciferase gene reporter under the control of the murine *hsp70-1* promoter (460 bp proximal). After identification of founders, breedings were carried out to successfully establish the transgene in the homozygous condition in 2 lines (TgN(HSE460w)Hel 101, 89). Transgenic 101 and 89 lines have integrated 20 and 55 copies of the transgene, respectively. Hemizygous animals used in the experiments were obtained by mating C57BL/6J females with transgenic homozygous males. Mice were housed in temperature- and humidity-controlled mouse room, maintained at 21°C with a 12-hour light-dark cycle. A commercial diet and water were supplied ad libitum before and during the experiments. The institutional health animal committee (University of Liege) approved the study.

In vivo treatments

For kinetic study of *hsp70-1* ribonucleic acid (RNA) production and luciferase activity after heat shock, forty 7-week-old female transgenic mice from line no. 101 were subjected to heat shock at 48°C for 15 minutes in a thermostatic chamber. Mice were killed by cervical dislocation 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 7 hours, or 9 hours after heat shock, and the lungs were collected for analysis. Eight additional sham-treated transgenic mice were handled in an identical manner, but they were maintained at room temperature. To evaluate the influence of age, sex, and transgenic lines on heat shock response of HSP70.1Luc, similar heat shock treatments were performed with male and female mice aged 6 weeks, 8 weeks, and 10 weeks from lines nos. 89 and 101 (3-6/group). Mice were killed to collect the lungs that were removed 4 hours after heat shock.

To investigate *hsp70-1* response to toxic exposure, we used 7-week-old female transgenic mice from line no. 101. The lungs were collected at different time intervals after treatment as mentioned in figure legends. Mice were exposed to 1 ± 0.22 ppm or 2 ± 0.35 ppm O_3 for 5 hours according to a protocol adapted from Delaunois et al (1998) and using the same equipment. Mice were placed in 17.5-L glass box supplied with filtered air enriched with O_3 with airflow of 2 L/min. O_3 concentration was continuously monitored by toxic gas analyzers. Control mice were exposed to filtered air without O_3. Administration of cadmium, paraquat, and parathion was performed as follows: mice were weighed and injected intra-peritoneally (ip) with $CdCl_2$ (5 mg/kg) or methyl paraquat (20 mg/kg or 70 mg/kg) in saline with an injection volume of 10 mL/kg body weight or with parathion-ethyl (1 mg/kg or 5 mg/kg) in a solution of ethanol (20%), propylene glycol (30%), and saline (50%), or the same volume of vehicle.

Additional mice were treated in the same way for functional and cytological evaluation by plethysmography and by bronchoalveolar lavage (BAL) fluid analysis ($n = 3$ to 5 by treatment) (see below).

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hsp70-1 gene expression analyzed by SYBR Green Real-Time Quantitative RT-PCR

Lungs samples were immediately snap-frozen in liquid nitrogen and stored at -80°C until analysis. RNAs were extracted by suspending pulverized tissue in RNA InstaPure reagent (Eurogentec, Liège, Belgium). One microgram of total RNA was reverse transcribed using oli-go-p$(dT)_{15}$ primer with an avian myeloblastosis virus reverse transcriptase (first strand cDNA synthesis kit for reverse transcriptase-polymerase chain reaction [RT-PCR], Roche, Indianapolis, IN, USA) and 1/25 of the cDNA was used in each PCR reaction. The same reaction was carried out without enzyme (RT-). In negative control, water replaced cDNA.

Specific primers for the murine *hsp70-1* gene (GenBank accession no. M35021) were chosen. The sense primer was 5'-TTGTCCATGTTAAGGTTTTGTGGTATA-3' and the antisense primer was 5'-GTTTTTTTCATTAGTTTGTAGT-GATGCAA-3'. The 18S ribosomal RNA (rRNA) was measured for each sample. PCR amplification was performed using the SYBR Green PCR master mix (for *hsp70-1)* or the TaqMan universal PCR master mix (for 18S rRNA) (PE Applied Biosystems) according to the manufacture's protocol. The thermal cycling conditions included 2 minutes at 50°C, 10 minutes at 95°C, and then 40 cycles of amplification for 15 seconds at 95°C and 1 minute at 60°C. The ABI PRISM 7700 Sequence Detection System instrument (PE Applied Biosystems) was used to measure fluorescence in real time. In a given sample, signals obtained for *hsp70-1* messenger RNA (mRNA) were normalized to the signal obtained for the 18S rRNA, and results were expressed as arbitrary units (fluorescence normalized value). The specificity of PCR amplifications was checked by 2% agarose gel electrophoresis.

Luciferase assay

Lung samples were frozen at -20°C until assayed. Measurement method was described elsewhere (Christians et al 1995). A half-lung was mixed in 150 µL of reaction buffer and centrifuged at 10 000 × *g* for 3 minutes. Twenty-five microliters of supernatant was diluted 1:1 with distilled H_2O. Light emission was integrated for 5 seconds at 23°C in a luminometer (Lucy 1, Anthos, Salzburg, Austria), and results were expressed as relative light units (RLUs). Background levels measured on the lungs from nontransgenic mice never exceeded 50 ± 10 RLUs. Therefore, 60 RLUs were subtracted from each sample measurement before calculating the luciferase activity per microgram of protein. The protein concentration in the supernatant was determined by bicinchoninic acid protein assay (Pierce, Rockford, IL, USA) with bovine serum albumin (BSA) as a standard. Intra- and interassay study was performed on luminometer by measuring dilutions of 3 lung samples. Each dilution was loaded 6 times on a same plate, 3 times on 3 different plates, and 3 times per plate for 5 days.

Assay with a standard curve using known dilutions of purified luciferase added in buffer was performed and showed that the signal was detectable from 10 fg of luciferase. Under these conditions, 10 RLUs corresponded to 2.5 fg of luciferase. In contrast, added in lung extract of control mice, the standard curve of luciferase showed that, in lung tissue matrix, the signal was detectable from 100 fg, and 10 RLUs corresponded to 22 fg of luciferase.

Hsp70 expression analyzed by Western blotting

Lung samples were homogenized in cold buffer (pH 7.4, 0.3 M sucrose, 0.03 M nicotinamide, 0.02 M ethylenedi-aminetetraacetic acid). Extracts were centrifuged (700 × *g* 10 minutes, 4°C and then 10 000 × *g* 5 minutes, 4°C). Supernatants containing cytosolic proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, according to Laemmli (1970). Proteins were transferred onto Immobilon-P membranes at 100 V (constant voltage, 1 hour) using Mini Trans-Blot electropho-retic transfer cell (Bio-Rad, Hercules, CA, USA). The blots were incubated with 50 mL of 5% non-fat-dried milk (w/v) for at least 1 hour and then washed and incubated for 1 hour at room temperature with rabbit anti-Hsp70 (stressgen, Spa812 diluted 1:10 000 in TBST containing 0.2% BSA). The blots were washed and then incubated in horseradish peroxidase-labeled goat anti-rabbit IgG (diluted 1:10 000 in Tris-buffered saline with 0.1% Tween-20 (TBST) containing 0.2% BSA) for 1 hour at room temperature. After the blots were washed, the immunocom-plexes were visualized with an enhanced chemilumines-cence detection kit. Then, the membrane was stained with Coomassie blue R-250 (Bio-Rad) according to current protocol to test for equal protein loading in each lane.

Plethysmography

Breathing of mice was studied with a whole-body ple-thysmograph (Buxco, Sharon, CT, USA) in unrestrained single chamber for mice as described by Segura et al (1999). Briefly, each mouse is introduced into a Plexiglas chamber supplied with a constant airflow (1 mL/s), which does not alter the respiratory signals. By breathing, the

animal within the chamber creates pressure fluctuations that are measured using a differential pressure transducer connected to an amplifier. The box pressure signal is analyzed by a software to give an index of airway obstruction named "enhanced pause" (Penh) (Ha-melmann et al 1997), calculated for each respiratory cycle. The values used in our study were obtained from the average of 10 minutes. The time interval between the end of the treatment and the measurement of Penh was chosen for each type of stress based on the peak of response determined during preliminary studies. These different time points were as follows: 2 hours after heat shock (48°C for 15 minutes), 22 hours after cadmium (5 mg/ kg), 24 hours after O_3 (2 ppm for 6 hours), 48 hours after paraquat (50 mg/kg), and 1 hour after parathion (5 mg/ kg). From exposure to stress until euthanasia for tissue collection, animals were observed regularly to detect any behavioral change.

Bronchoalveolar lavage

Immediately after plethysmography, the mice were deeply anaesthetized with pentobarbital (100 mg/kg ip) and killed by transecting the renal artery. BAL was performed to count total and differential cell number according to Watkinson et al (1996).

Statistical analysis

Values were averaged in each group and compared between treated and control groups. Data are expressed as means ± standard error of mean. A Student's t-test for paired or unpaired samples was used as appropriate for comparison of 2 means. Differences were considered as significant when $P < 0.05$. The statistical effects of age, sex, and line of animals on transgene expression were assessed by 2-factor analysis of variance (ANOVA 2).

RESULTS

Heat shock response in HSP70.1Luc transgenic mouse model

The HSP70.1Luc transgene includes the firefly luciferase reporter gene under control of the proximal part of the murine *hsp70-1* gene promoter. Heat-induced expression of similar transgenes using a longer regulatory region has been analyzed previously in embryonic cells (Thompson et al 1994; Christians et al 1995). But the time course of the stress response was never investigated in adult lungs; therefore, we started our study by characterizing the expression of both endogenous *hsp70-1* gene and HSP70.1Luc transgene in the lungs after heat shock exposure (Fig 1 A,B). Induction of endogenous *hsp70-1* gene was analyzed by SYBR Green Real-Time Quantitative RT-PCR (Fig 1A). In control mice, the lungs expressed basal level of *hsp70-1* mRNA, indicating that the endogenous *hsp70-1* was constitutively expressed at a low level at normal temperature. Induced transcription of *hsp70-1* gene in lungs appeared 30 minutes after heat shock exposure, reaching a maximum level within 1-3 hours later. Level of induction of the transgene HSP70.1Luc was analyzed by measuring the luciferase activity (Fig 1B). No basal activity could be detected in lungs in the absence of stress. Induced activity was observed as early as 2 hours after heat shock and lasted 7 hours before returning to the background level. A peak was observed between 3 hours and 5 hours after heat shock. Comparing these 2 sets of data, it appeared that luciferase activity showed a time course similar to the endogenous gene transcripts with a right shift of 2 hours.

Regarding male and female differences, we noticed that males of over 10 weeks of age expressed higher levels of luciferase than did females (11.1 ± 4.4 vs 1.4 ± 0.4 RLU/µg of protein) (ANOVA 2: $P < 0.05$) and that males were affected more than the females by the heat shock treatment because 40% of the males (6/15) died during heat shock exposure. Transgene expression analyzed in animals from 6 weeks to 12 weeks did not show any age-related difference. Copy number or integration sites, which differ among transgenic line, did not affect the profile of transgene expression. Taking into account these results and the high sensitivity of the males, we decided to use 7-week-old females from line 101 in further experiments.

In addition, an intra- and interassay study was carried out (see Material and Methods) to confirm that our measurement method was not responsible for the variability observed between mice (intraassay CV = 9.8%, interassay CV = 11.3%).

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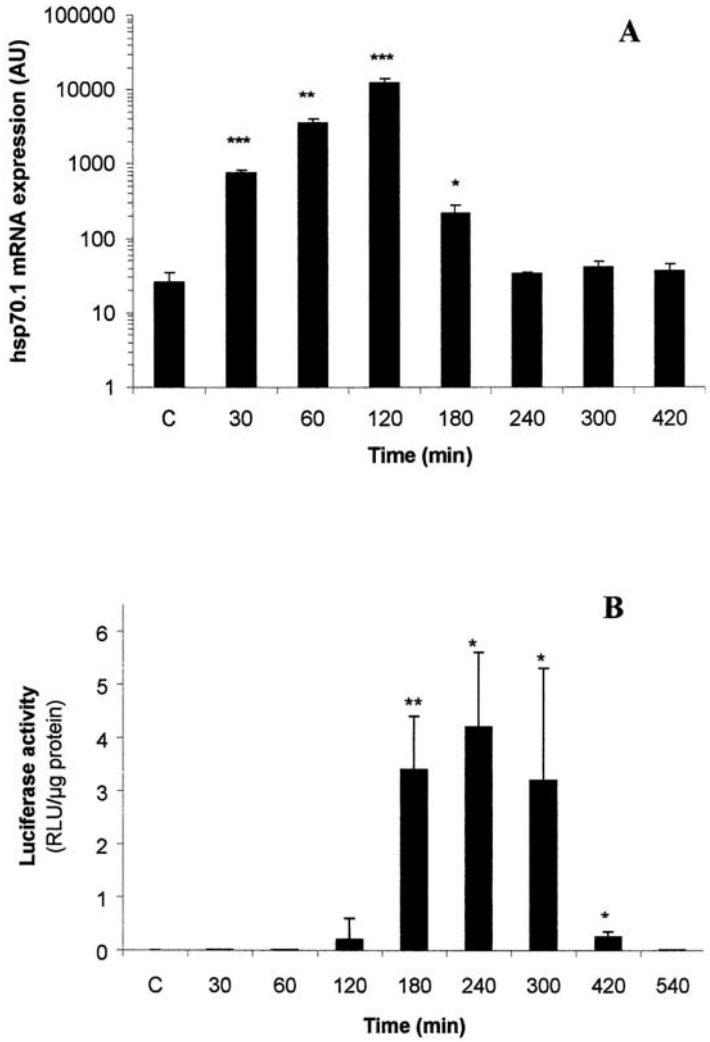
Fig. 1: *Kinetics of HSP70.1 induction by heat shock (48°C for 15 minute). (A) Analysis of endogenous hsp70-1 mRNA was evaluated by SYBR Green Real-Time Quantitative RT-PCR. Level of mRNA expression is expressed as arbitrary units (AU) corresponding to the ratio (RT-PCR signal for the Hsp70.1 mRNA)/(RT-PCR signal for the 18S rRNA). Two animals were analyzed for each time period tested in duplicate. Values represent the means ± SE. (B) Analysis of HSP70.1LUC expression in lung was assessed by measurement of luciferase activity (RLU/µg of protein) in tissue extracts. Each value represents the means ± SE calculated from measurements made with n = 5 mice for each time period tested. (*, P < 0.05; **, P < 0.01; ***, P< 0.005). mRNA, messenger ribonucleic acid; RT-PCR, reverse transcriptase-polymerase chain reaction; rRNA, ribosomal RNA; SE, standard error; RLU, relative light unit.*



hsp70-1 response to toxic exposure

To investigate the *hsp70-1* response to various environmental and chemical stresses, we subjected transgenic mice to acute exposure to several pollutants (cadmium, O_3, parathion, paraquat) and measured transgene activity (Fig 2). The sublethal concentrations used for toxic compounds were in the range used by other authors in toxicological studies using mice or rats (cadmium [5 mg/kg, ip], O_3 [1 ppm or 2 ppm, 5 hours], paraquat [20 mg/kg or 70 mg/kg, ip], and parathion [1 mg/kg or 5 mg/kg, ip]) (Weitman et al 1986; Goering et al 1993; Nakanishi and Yasumoto 1997; Currie et al 1998). The lungs were sampled at various time intervals after administration. Transgene activity driven by *hsp70-1* promoter was clearly increased after cadmium administration. Maximum level of induction was observed 10 hours after exposure. Acute exposure to the other pollutants (O_3, parathion, paraquat) did not induce any increase in luciferase activity, suggesting that the transgene does not significantly respond to such type of stress.

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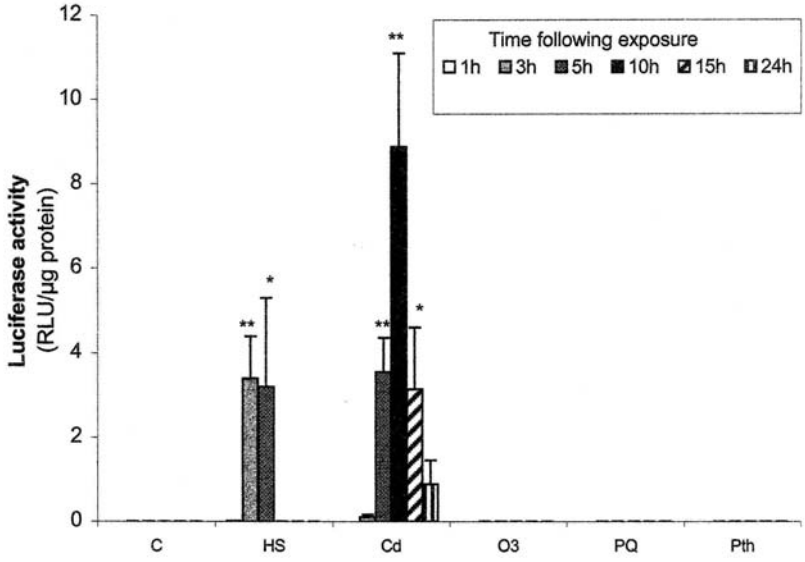
Fig. 2: *HSP70.1LUC response to toxicant exposure. Mean luciferase activity levels (RLU/µg protein) ± SE in the lungs of transgenic mice sampled at various times after exposure to various stresses at the indicated doses. C, control; HS, heat shock (48°C, 15 minute); Cd, cadmium chloride (5 mg/kg); O_3, ozone (1-2 ppm); PQ, methyl paraquat (20-70 mg/kg); Pth, parathion-ethyl (1-3 mg/kg). Measurement was made in 3 animals for each time point and treatment. (*, P < 0.05; **, P < 0.01). RLU, relative light unit; SE, standard error.*



To further investigate possible discrepancy between transgenic and endogenous *hsp70-1* promoters activity, the lungs from heat shocked, toxicant-exposed, and control mice were harvested to investigate *hsp70-1* transcription by SYBR Green Real-Time Quantitative RT-PCR (Fig 3). The amplified RT-PCR product of *hsp70-1* was analyzed by agarose gel, and it displayed the expected size. Contamination by residual genomic DNA was excluded as no *hsp70-1* amplification was detected in wells loaded with corresponding RT-PCR products where RT has been omitted (data not shown). Amplification of the housekeeping 18S rRNA was positive in all samples subjected to RT and used as positive control, indicating relative quantity of amplified cDNA for each sample. As observed with the measurement of the HSP70.1Luc transgene expression, Real-Time RT-PCR indicated that the *hsp70-1* endogenous gene was activated by cadmium but not by O_3, paraquat, or parathion exposure. Together, these results confirm that both transgene and endogenous *hsp70-1* promoters responded in a same way to external stimuli, as already observed after heat exposure. In addition, these confirm that *hsp70-1* gene is induced by a part of, but not all, the inflected stresses.

Investigations mentioned above had specifically analyzed *hsp70-1* activity. Although *hsp70-1* is the major inducible *hsp70*, we could not rule out the possibility that the other members of that family might be affected by the different toxicants we were testing. Because several studies reported Hsp70 synthesis in lungs after O_3 treatment (Wong et al 1996; Su and Gordon 1997), we decided to reexamine the lung response at the level of Hsp70 protein synthesis after O_3 exposure, using cadmium as positive control (Fig 4). O_3 did not alter significantly the amounts of expressed Hsp70, whereas after cadmium treatment, the synthesis of Hsp70 was markedly induced.

Functional and cytological responses in lungs exposed to stress

To investigate the relationship between the stress response (estimated in lungs by measurement of *hsp70* induction) and functional or lesional change, plethysmography and BAL fluid analysis were performed after heat shock or toxicant exposure (Table 1). Penh, an index of airway obstruction measured in nonanesthetized and freely moving mice, was slightly increased after cadmium and markedly increased in mice exposed to O_3, paraquat, or parathion, suggesting that these toxicants exerted an effect on the respiratory mechanics. In addition, BAL fluid analysis revealed a significantly higher cell count in the lungs exposed to O_3 and paraquat than in controls. This was mainly because of an increased number of neutrophils. In contrast, when mice were exposed to heat shock, no modification was observed in Penh and no change in cytological composition of BAL fluid after heat shock or cadmium. Finally, behavioral changes such as agitation after heat stress or apathy and several signs of poisoning after cadmium, paraquat, or parathion administration were noted. Whereas heat shock or

cadmium exposure led mainly to clinical changes rather than to pulmonary disturbances, O_3, paraquat, or parathion induced changes in respiratory mechanics and a pulmonary inflammatory phenomenon. Nevertheless, these observations showed that all the pollutants used in our study induce a physiopathological stress for mice lungs, although this physiopathological stress does not necessarily provoke a stress response based on overexpression of Hsp70.

Fig. 3: *Quantitative analysis of hsp70-1 gene response to toxicant exposure. hsp70-1 transcripts in mouse lungs were analyzed by SYBR Green Real-Time Quantitative RT-PCR after HS, heat shock (48°C, 15 minute); Cd, cadmium chloride (5 mg/kg); O_3, ozone (1 ppm); PQ, paraquat (20 mg/kg), or Pth, parathion (1 mg/kg) exposure. Lungs were harvested at 1 hour, 3 hours, or 5 hours after toxicant exposure. Data represent mRNA level expressed as arbitrary units (AU) corresponding to the ratio (RT-PCR signal for the Hsp70.1 mRNA)/(RT-PCR signal for the 18S rRNA). Two animals were analyzed for each time period tested in duplicate. Values represent the means ± standard error. (*, P < 0.05; **, P < 0.01 ; ***, P < 0.005). The specificity of PCR amplifications was checked by 2% agarose gel electrophoresis. RT-PCR, reverse transcriptase-polymerase chain reaction; mRNA, messenger ribonucleic acid; rRNA, ribosomal RNA.*

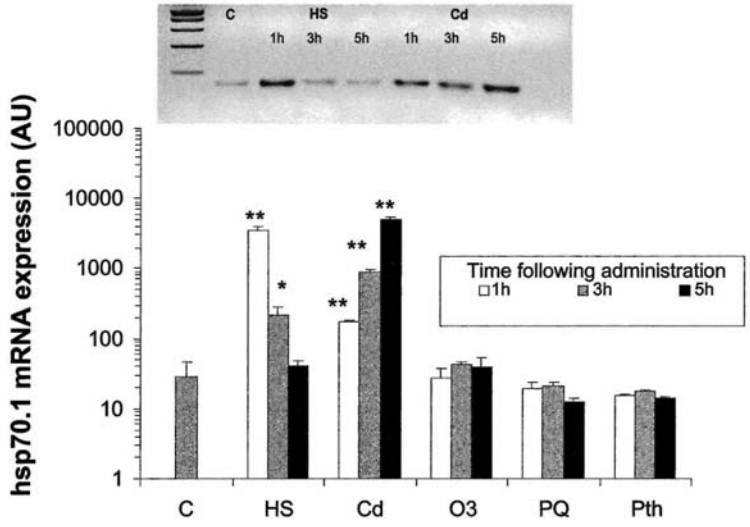


Fig. 4: *Western blot analysis of Hsp70 protein in mouse lungs. Hsp70 proteins were evaluated 24 hours after ozone (1 ppm) or cadmium (1 mg/kg) exposure. O_2, air exposed mice; O_3, ozone exposed mice; NaCl, control mice injected with NaCI 0.9%; Cd, cadmium treated mice.*

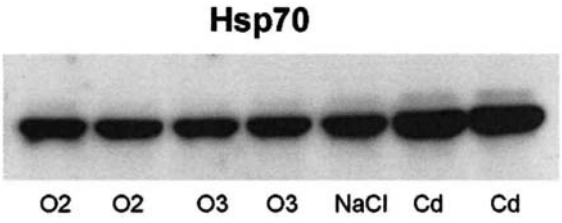


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Table 1: *Effects of different stress exposures on behavior of mice, enhanced pause (Penh), and the number of bronchoalveolar lavage (BAL) fluid cells*

Stress	Recovery period	n[a]	Symptoms[b]	Penh[c]	BAL cells (Total cells/mL [×10^3]) (Neutrophiles/mL [×10^3])
Controls	24 h	4	—	0.46 ± 0.05	Total cells: 92.5 ±11.9 Neutrophils: 2.08 ± 0.5
Heat shock (48°C, 15min)	2 h	3	Sweating Gasping Increased activity	0.54 ± 0.02	Total cells: 94.4 ±12.9 Neutrophils: 2.36 ± 0.5
Cadmium (5 mg/kg, ip)	22 h	4	Apathy Ophthalmic secretion	0.95 ± 0.05*	Total cells: 97.1 ± 10.4 Neutrophils: 2.6 ± 0.4
Ozone (2 ppm, 6 h)	24 h	3	Increased amplitude of breathing	2.46 ± 0.06**	Total cells: 138.7 ±11.1* Neutrophils: 10.26 ± 1.67**
Paraquat (50 mg/kg, ip)	48 h	5	Apathy Increased amplitude of breathing	4.33 ± 0.18**	Total cells: 151.7 ±17.2* Neutrophils: 30.6 ± 2.12**
Parathion (5 mg/kg, ip)	1 h	4	Apathy Transient paralysis Diarrhea Salivation	1.12 ± 0.09**	Total cells: 90.6 ± 8.9 Neutrophils: 2.17 ±0.36

[a] Number of tested mice.
[b] Observed during the recovery period.
[c] Index of airway obstruction measured in whole-body plethysmograph. Values are means ± standard error.
* Significant value compared with controls (*, $P < 0.05$; **, $P < 0.01$).

DISCUSSION

The purpose of our study was to design and exploit an in vivo bioassay to further investigate the hypothesis, suggested by Wong and Wispe (1997), that Hsp70 response is stimulus-dependent in the lungs. To easily follow *hsp70-1* gene activation by various toxicants, we used a HSP70.1Luc transgenic model similar to those described previously (Christians et al 1995), and we defined some of its limits in vivo. Furthermore, we showed that the response exhibited by the Hsp70 was differential according to stress because it was activated by heat or cadmium exposure but not by O_3, paraquat, and parathion, though each of the last three made a significant impact on the lungs.

Initially, we have characterized the transgene response in lungs using a thermal stress as a reference for *hsp* induction. As expected, the lungs of transgenic mice showed a clear response by overexpressing endogenous *hsp70-1* gene as well as the luciferase hybrid transgene (Fig 1 A,B). It is worth mentioning that the 2-hour difference observed between the expression peaks of the *hsp70-1* mRNA and the protein luciferase is consistent with the time-lag that other studies have described between Hsp70 transcription and synthesis; proteins accumulate until the mRNA returns to the control level (Wagner et al 1999). As previously observed (Blake et al 1990; Tanguay et al 1993), the lungs from control mice constitutively expressed a basal level of *hsp70-1* mRNA (see Figs 1A and 3), which was higher in pulmonary tissue than in heart (data not shown). Several explanations can account for the absence of basal level of luciferase activity in lungs. It is possible that the proximal portion of the *hsp70-1* promoter we used in the transgene does not include the required sites for constitutive expression. A second possibility is that the chromatin organization represses the activity of the transgene unless a stress is applied. Finally, we have noticed a reduced sensibility of the luciferase assay when it is conducted on tissue extracts in comparison with in vitro assay (see Material and Methods).

To further investigate in vivo the stimulus dependence of Hsp70 response in lungs, we challenged HSP70.1Luc transgenic animals with toxicants belonging to different chemical families and measured the transgene activity. Cadmium strongly induced HSP70.1Luc from 5 hours to 15 hours after exposure (Fig 2). Our observation that HSP70.1Luc response was delayed, and more sustained after cadmium in comparison with heat shock, likely correlates with stress duration endured by cells after each treatment. Indeed, heat shock exposure is transient, whereas injected cadmium accumulates in tissues and cells, thus, its toxic effects last longer (Swiergosz-Kowalewska 2001). Although other studies have shown that pollutants, such as O_3, and pesticides could also induce Hsps expression in the lungs and in the liver, respectively (Bagchi et al 1996; Nakanishi and Yasumoto 1997; Su and Gordon 1997), the transgene was not induced by O_3, paraquat, or parathion at any of the time-point tested. Lack of HSP70.1Luc response in our study was not artefactual as confirmed by the absence of endogenous *hsp70-1* response (Fig 3). It is difficult to explain the discrepancy with other studies because model used, gene investigated, or pollutants tested are not similar. In our study, we specifically analyzed *hsp70-1* , which is the

major inducible Hsp70 gene but belongs to a family that includes other members (Hunt and Calderwood 1990). Therefore, to evaluate the participation of other *hsp70* genes in the response observed in these other studies, we analyzed Hsp70 expression by Western blot in the case of O_3 exposure, which was the most debated (Su and Gordon 1997; Wu et al 1999). Amount of Hsp70 was not increased, confirming that O_3 treatment we used was not able to induce Hsp70 synthesis in the lungs (Fig 4).

In addition, although Wong and Wispe (1997) suggested the possibility that the stimulus dependence is lung specific, we observed that in the heart, liver, and kidneys, HSP70.1Luc transgene response to heat shock, cadmium, 0_3, paraquat, or parathion was similar to that in the lungs (data not shown). This leads us to conclude that the stress dependence of *hsp70-1* induction is not specific to lung in our model but rather widely observed in various tissues.

Correlation between toxicity and ability to induce Hsp70 overexpression has been suggested for physical or chemical agents in various models (Goering et al 1993; Rajdev and Sharp 2000). But real evidence or precise investigations about efficiency of "heat shock response" to discriminate toxicity between different agents have never been reported. In our study, functional and cellular alterations were assessed in parallel to the induction measurements. We observed that whereas heat shock or cadmium exposure induced no specific pulmonary disturbances, O_3, paraquat, or parathion led to changes in respiratory mechanics and lung inflammatory response (Table 1). These results reveal that the stress dependence of *hsp70-1* in the lungs is not correlated with toxicity degree of the stressors.

From these data, it seems clear that *hsp70-1* activation is related to the mechanisms by which the different toxicants cause alterations in the lungs rather than the intensity of their toxic effects. Then, the following question is to identify the difference between the compounds used in our study, knowing that all of them can cause an elevation in oxidative stress level. Cadmium leads to changes in oxidized and reduced glutathione pools, and its high affinity for thiol groups causes the formation of non-native disulfide bonds, resulting in the accumulation of misfolded proteins, the signals triggering the heat shock response (Stohs and Bagchi 1995; Zou et al 1998; Stevens et al 2000). O_3 induces oxidative stress injury mainly by peroxidation of membrane lipids; protein oxidation is caused by secondary reactions (for review see Mudway and Kelly 2000). In a similar way, in cells, paraquat exposure provokes lipid peroxidation after reduced nicotinamide adenine dinucleotide phosphate-dependent reduction and production of superoxide anion (Smith 1987). Although lipid peroxidation was described after organo-phosphate exposure (Bagchi et al 1995), oxidative mechanism of parathion was not extensively studied. Taking together our data and those discussed above, it seems that the agents causing oxidative injury to the lungs can elicit different patterns of stress responses, as suggested by Timblin et al (1998). We propose that Hsp70 expression is stimulated by those that are primarily responsible for protein alteration, whereas other compounds that are damaging indirectly the protein structure do not systematically activate *hsp70* transcription or even can affect and repress heat shock factor activation. This last alternative remains to be further investigated.

Further studies with larger series of toxicants may lead to better characterize the biological and biochemical signals involved in the stimulus dependence of the Hsp70 response found in this study. The simplicity of the luciferase assay should allow the use of our model in screening programs for the detection of specific Hsp70 inducers.

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