Physiol. Res. 55: 405-411, 2006

Effect of Chronic Hypoxia on Proliferation, Apoptosis, and HSP70 Expression in Mouse Bronchiolar Epithelial Cells

E-K. KIM, J.D. PARK, S-Y. SHIM, H-S. KIM, B.I. KIM, J-H. CHOI, J.E. KIM[1]

Department of Pediatrics, [1]Department of Pathology, Seoul National University College of Medicine, Seoul, Korea

Received February 22, 2005
Accepted August 10, 2005
On-line available October 17, 2005

Summary

Heat shock proteins (HSPs) can be induced by various stresses and play an important role in cell cycle progression. HSP70 has been shown to act as an inhibitor of apoptosis. We studied HSP70 expression in bronchial epithelial cells of C57BL/6 mice and homozygous HPS70 knockout mice (*hsp70.1*$^{-/-}$) exposed to chronic hypoxic stress. We also investigated changes in cellular proliferation and apoptosis in relation to HSP70. Lungs were removed from mice after a three-week period of exposure to 10 % O_2. Immunoblots for HSP70 and immunohistochemical staining for HSP70 and Ki-67 were performed. Apoptosis was assessed using the TUNEL assay. The three-week period of hypoxic stress did not change HSP70 levels in total lung tissue, but a significant reduction in HSP70 expression was observed in bronchiolar epithelial cells. In wild type mice, both HSP70 and Ki-67 expression were significantly reduced in bronchiolar epithelial cells. In homozygous HPS70 knockout mice (*hsp70.1*$^{-/-}$), apoptosis of bronchiolar epithelial cells was significantly increased. Our results suggest that HSP70 may exert anti-apoptotic effects in mouse bronchiolar epithelial cells.

Key words

Chronic hypoxia • Bronchiolar epithelium • HSP70 • Proliferation • Apoptosis

Introduction

Heat shock proteins (HSPs) are present in prokaryotic and eukaryotic cells. Their highly conserved structure suggests that they play a crucial role in cellular processes. Heat shock is not the only stimulus that can induce and increase the synthesis of HSPs. Exposure of cells to amino acid analogs (Kelly and Schlesinger 1978), glucose analogs (Pouyssegur *et al.* 1977), heavy metals (Levinson *et al.* 1980), protein kinase C stimulators (Ding *et al.* 1996), Ca^{2+}-increasing agents (Ding *et al.* 1996), ischemia, sodium arsenite (Johnson *et al.* 1980), microbial infections, nitric oxide, hormones or antibiotics can also induce the expression of HSPs.

Mammalian HSPs are classified into four major families according to their molecular size: HSP90, HSP70, HSP60 and the small HSPs. The HSP70 family constitutes the most conserved and the best studied class and includes both cognate members and highly inducible isoforms. Under normal conditions HSP70 proteins function as ATP-dependent molecular chaperones. Under various stress conditions, the synthesis of stress-inducible HSP70 enhances the ability of cells to cope with increased concentrations of unfolded or denatured

© 2006 Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic
Fax +420 241 062 164
E-mail: physres@biomed.cas.cz http://www.biomed.cas.cz/physiolres

proteins (Nollen *et al.* 1999). HSP70 also increases the tumorigenicity of cancer cells in rodent models (Jäättelä 1995). Moreover, HSP70 can inhibit apoptosis and thereby increase the survival of cells exposed to a wide range of lethal stimuli (Jäättelä *et al.* 1992, Mosser *et al.* 1997).

A number of studies have shown that HSPs play important roles in cell cycle progression. For example, overexpression of human HSP72 stimulates the growth rate and appearance of S-phase cells in the SHOK cell line (Suzuki and Watanabe 1994). Evidence for a role of HSP70 in cell proliferation has also been obtained from *hsp70* antisense application, which abolished progress through the G1- and S-phases of the cell cycle in human tumor cells (Wei *et al.* 1995).

Hypoxia is a fundamental stressor to living organism and many kinds of physiological or pathological processes are induced by hypoxia. The lung is the primary organ that is directly exposed to the hypoxic environment. Little is known about how lung airway cells respond to chronic hypoxia in terms of the production of HSPs. Heat shock pretreatment protects thyroid FRTL-5 cells from hypoxia, suggesting that HSPs play an essential role in the protection from hypoxic injury (Kiang *et al.* 1996). The goal of the present study was to examine the HSP70 response of lung airway cells of mice to chronic hypoxia. Cellular proliferation and apoptosis in relation to HSP70 were also investigated. To further define the role of HSP70 in chronic hypoxic stress, the same experiments were carried out on homozygous HSP70 knockout mice (*hsp70.1*$^{-/-}$).

Methods

Animals

Seven-week-old male C57BL/6 mice (n=20) were randomly divided into a normoxic group and a hypoxic group. Hypoxia (10 % O_2) was produced by O_2 dilution under normobaric conditions using a mixture of air and nitrogen. After three weeks, animals were killed and the lungs were excised and prepared for immunoblot and immunohistochemical studies. Homozygous HSP70 knockout mice (*hsp70.1*$^{-/-}$) were also randomly divided into a normoxic group and a hypoxic group, and the same experiments were conducted on these animals.

Construction of hsp70.1 targeting vector and generation of knockout mice

A murine genomic clone of the *hsp70.1* locus was cloned from a λ FixII phage library prepared from 129/Sv embryonic stem cells using a human *hsp70* cDNA probe and characterized by Southern blot analysis and DNA sequencing. The targeting vector contained a 7.5 kb *Not*I–*Xho*I fragment from the 5′ promoter and a regulatory region of the *hsp70.1* gene as the long arm, a 1.8 kb neomycin-resistant gene, a 0.8 kb *Not*I-*Sma*I fragment derived from the *hsp70.1* exon as the short arm, and a 3.4 kb fragment containing two copies of the herpes simplex virus thymidine kinase gene. Some coding sequences of the promoter were deleted and replaced by a PGK-neoexpression cassette. Ten micrograms of *Not*I-linearized targeting vector was electroporated into E14/BK4 embryonic stem cells and correct targeted clones were selected with G418 (0.2 mg/ml; Life Technologies, Gaithersburg, MD) and FIAU (200 μM, Syntex, Palo Alto, CA) in DMEM medium. Three independent homologous recombinant *hsp70.1* embryonic stem cell clones were injected into C57BL/6 blastocysts and heterozygous mutant mice were generated from one cell line. The mutant mice used in all experiments were backcrossed onto the C57BL/6 strain for four generations.

Immunoblot analysis

The entire unilateral lung from each animal in the four groups was homogenized, centrifuged at 20 000 × *g* and suspended in sodium dodecyl sulfate-glycerol. Immunoblotting was performed with 30 μg of total protein per lane. Blots were labeled with a primary polyclonal rabbit anti-human HSP70 antibody (Stressgen, Canada) and then exposed to a goat anti-rabbit secondary antibody (Santa Cruz, USA). The signal was detected with enhanced chemiluminescence (Amersham Pharmacia Biotech) and the density of chemiluminescence was quantified using scanning densitometry.

Immunohistochemistry

HSP70

Immunohistochemistry was performed as previously described (Weiss *et al* 2002). The primary polyclonal rabbit anti-human HSP70 antibody (Stressgen, Canada) was used at a 1:100 dilution. Sections were then incubated in secondary goat anti-rabbit antibody, conjugated to peroxidase-labeled dextran polymer (Envision+, DAKO, USA), 3,3-diaminobenzidine tetrahydrochloride was then added and counter-staining with Mayer's hematoxylin was performed. Staining was

Table 1. Response of seven-week-old wild type and homozygous knockout mice to chronic hypoxia

Genotype	+/+		–/–		
Condition (n)	Normoxia (10)	Hypoxia (10)	Normoxia (10)	Hypoxia (10)	*P*-value
HSP70, BE (grade 0–3)	2.10 ± 0.23	0.80 ± 0.20	1.00 ± 0.30	0.90 ± 0.31	0.010
Ki-67, BE %(+) cells	2.40 ±0.28	1.25 ± 0.15	1.97 ± 0.23	2.40 ± 0.19	0.004
TUNEL, BE %(+) cells	0.40 ± 0.16	1.30 ± 0.50	1.70 ± 0.26	2.20 ± 0.25	0.003

+/+ : *hsp70.1(+/+),* wild type mice*;* –/– : *hsp70.1(–/–),* homozygous knockout mice. BE: bronchiolar epithelium, NS: not significant. Data are means ± S.E.M. *P*-values are from Kruskal-Wallis test comparing the four groups.

then graded semi-quantitatively using grades 0 to 3 (0: negative; 1: weak positive; 2: moderate positive; 3: moderate to strong positive) by a pathologist who did not have information about the experimental groups.

Ki-67

The Ki-67 protein is present during all active phases of the cell cycle (G_1, S, G_2, and mitosis), but is absent in resting cells (G_0). The fact makes it an excellent marker for proliferating cells (Scholzen and Gerdes 2000). The immunohistochemical detection of Ki-67 was performed to evaluate the proliferation indices of the bronchiolar epithelium. Primary rat anti-mouse Ki-67 antibody (DAKO, USA) was used at a dilution of 1:50. Biotinylated secondary anti-rat immunoglobulins (DAKO, USA) were used at a dilution of 1:500. The rest of the procedure was the same as that described for HSP70 immunohistochemistry. Labeled cells were counted and results were expressed as a percentage of total cells.

Apoptosis

Apoptotic cells were localized by *in situ* staining of DNA breaks in the nuclei of dying cells in tissue sections using a commercial kit (FragEL DNA Fragmentation Detection Kit, EMD Biosciences, USA) for the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labeling (TUNEL) method (Ben-Sasson *et al.* 1995). Labeled nuclei were counted and the results were expressed as a percentage of total nuclei.

Statistical analysis

All data were expressed as means ± S.E.M. The difference in the percentage of stained cells was analyzed using the Kruskal-Wallis test for the full data set and using the Mann-Whitney U test for group-by-group comparison. Correlations between the expression of HSP70 and the percentage of cells positive for Ki-67 were performed using the Spearman rank test. To find the best predictor of apoptosis, standard multiple regression analysis was used. The significance level was set at $P<0.05$.

Results

Immunoblot analysis

Immunoreactivity for the HSP70 protein in lung homogenates was same in the four groups of animals studied.

Bronchiolar epithelial cell HSP70 immunohistochemistry

HSP70-labeled bronchiolar epithelial cells were significantly fewer in the wild type hypoxia group (WH), the knockout (*hsp70.1*$^{-/-}$) normoxia group (KN), and the knockout (*hsp70.1*$^{-/-}$) hypoxia group (KH), compared with the wild type normoxia group (WN). The WH group showed the most apparent decrease (2.10±0.23 vs. 0.80±0.20: P=0.002), and the KN and KH groups also exhibited a significant reduction (1.00±0.30: P=0.015 and 0.90±0.31: P=0.019, respectively) (Table 1; Fig. 1).

Bronchiolar epithelial cell Ki-67 immunohistochemistry

The proliferative activity of the bronchiolar epithelium, as assessed by Ki-67 immunohistochemistry, was significantly lower in the WH group compared with the WN group (2.40±0.28 % vs. 1.25±0.15 %: P=0.003). In the WH group, the number of HSP70 and Ki-67-labeled cells was significantly lower (P=0.002 and 0.003

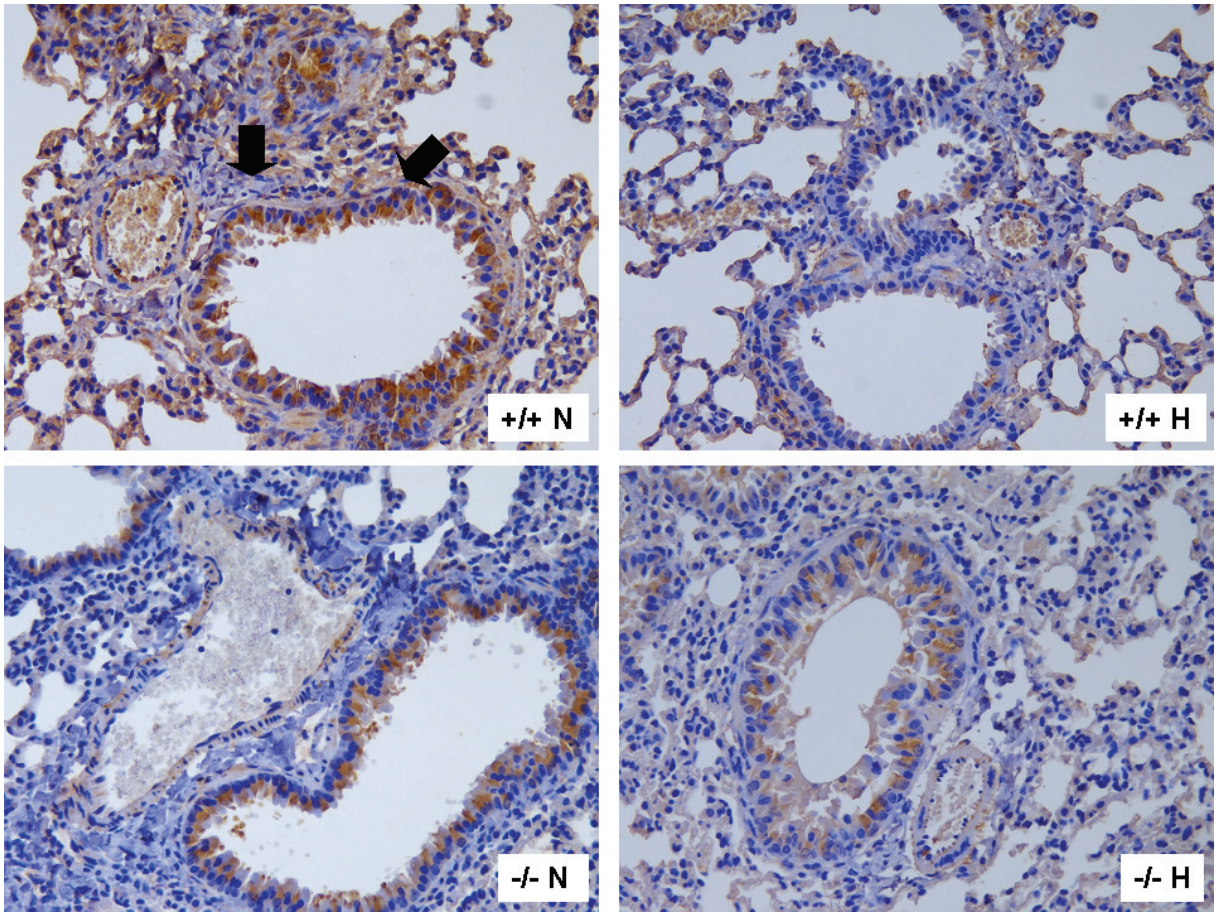

Fig. 1. HSP70 expression in bronchiolar epithelium. Immunohistochemical staining for HSP70 showing strong and diffuse immunoreactivity in wild type normoxia group (arrows). (+/+: *hsp70.1(+/+)*, wild type mice; –/–: *hsp70.1(–/–)*, knockout mice; N: normoxia; H: hypoxia).

respectively) compared with the WN group. However, no correlation was observed between these two variables. In the homozygous knockout (*hsp70.1*$^{-/-}$) mice, Ki-67-labeled cells were similar in number to the WN group, irrespective of oxygen conditioning (Fig. 2). Multiple regression analysis did not reveal any significant contribution to proliferation.

Bronchiolar epithelial cell apoptosis

The percentage of TUNEL-positive cell nuclei increased significantly in the KN group and the KH group compared with the WN group (0.40±0.16 % vs. 1.70±0.26 %: P=0.002; and 0.40±0.16 % vs. 2.20±0.25 %: P=0.000, respectively). The WH group showed a small increase in TUNEL-positive cell nuclei compared with the WN group, but statistical significance was not demonstrated. Additionally, the KH group did not show a significant increase in TUNEL-positive cell nuclei when compared with the KN group (Fig. 3). Standard multiple linear regression analysis revealed that the genotype, oxygen environment, and HSP70 expression accounted for 32.6 % of the variance in apoptosis. Of these three variables, the genotype made the largest contribution (beta=0.447) with statistical significance (P=0.003).

Discussion

Our results have shown that exposure to hypoxic stress for three weeks does not change HSP70 levels in total lung tissue, but a significant reduction of HSP70 expression occurs in bronchiolar epithelial cells. Using immunohistochemistry, we demonstrated that both HSP70 and Ki-67 expression are significantly reduced (P=0.002 and P=0.003, respectively) in bronchiolar epithelial cells by hypoxic stress in wild type mice. Furthermore, the knockout genotype (*hsp70.1*$^{-/-}$) significantly contributes to the apoptosis of bronchiolar epithelial cells.

HSP70 levels, measured from unilateral lung

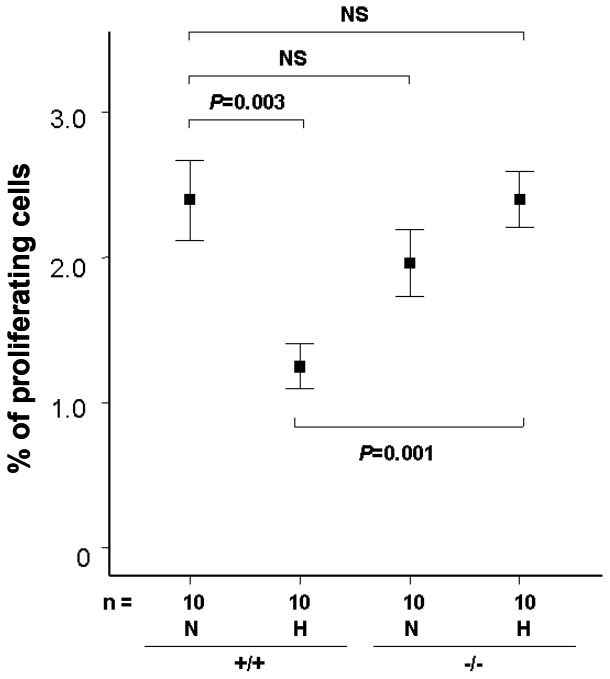

Fig. 2. The percentage of Ki-67-positive bronchiolar epithelial cells reduced significantly in the wild type hypoxia group compared with the wild type normoxia group (P = 0.003) (+/+: *hsp70.1(+/+),* wild type mice; –/–: *hsp70.1(–/–),* knockout mice; N: normoxia; H: hypoxia; NS: not significant).

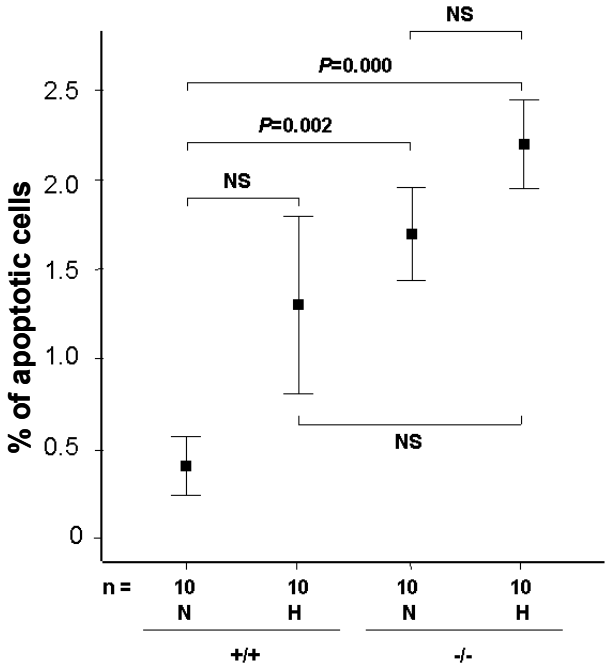

Fig. 3. The percentage of TUNEL-positive cell nuclei increased significantly in knockout (*hsp70.1*$^{-/-}$) normoxia group and the knockout hypoxia group compared with the wild type normoxia group (P = 0.002 and P = 0.000, respectively) (+/+: *hsp70.1(+/+)*, wild type mice; –/–:*hsp70.1(–/–)*, knockout mice; N: normoxia; H: hypoxia).

homogenate by the immunoblot method, did not differ among the four groups studied. Thus, chronic hypoxia did not change HSP70 expression in a total lung tissue homogenate. However, HSP70 expression was significantly reduced by chronic hypoxia in bronchiolar epithelial cells. The substantial expression of HSP70 in knockout (*hsp70.1*$^{-/-}$) mice may be due to the *hsp70.3* gene, another inducible HSP gene that encodes for HSP70.

It is generally known that the HSPs are increased during various kinds of stress, so our results can be qualified as surprising. Nevertheless, decreased HSP70 expression in acute stress and negligible changes in chronic stress have also been reported (Filipovic *et al.* 2005). Furthermore, electromagnetic field exposures which have been shown to induce HSPs diminished HSP70 levels after long-term continuous exposure (Di Carlo *et al.* 2002). The increase of HSP70 is reported to be transient, and its persistence is different in various cell types (Kiang and Tsokos1998). Thus, it is possible that HSP70 expression in bronchiolar epithelial cells in our experiments initially increased in response to hypoxia and then decreased below control levels after 21 days. Another explanation might be based upon increased turnover of epithelial cells, which did not permit the synthesis of sufficient quantities of HSP70. Reducing oxygen concentration has been demonstrated to increase the division rate and final density of fibroblasts cultured in serum-containing medium (Storch and Talley 1988). But, the hypobaric hypoxia induced different patterns of ^{3}H-thymidine uptake in different types of lung cells, and it was significantly above the control value on day 3, 5 and 7 and significantly decreased at day 14 in the alveolar walls (Meyrick and Reid 1979). The proliferative activity of the bronchiolar epithelium, as assessed by Ki-67 immunohistochemistry, was also significantly lower in the WH group compared with the WN group in our experiments. Thus, an increased turnover is not the likely mechanism. Mast cells increase in pulmonary arteries in chronic hypoxia and are suggested to be the key effector cells in mediating collagen turnover, thus play an important role in hypoxic pulmonary vascular remodeling and its regression (Riley *et al.* 2000). These cells may be involved in epithelial cells, but no significant change in periairway mast cell content in response to alveolar hypoxia was reported in contrast to a 12 % decrease in the granule content of perivascular mast cells in the same situation (Nadziejko *et al.* 1989).

In wild type mice, both HSP70 and Ki-67 expression were significantly reduced by chronic hypoxia

(P=0.002 and P=0.003, respectively). There is some evidence that HSPs play an important role in cell cycle progression, but we found no correlation between HSP70 and Ki-67 expression. The KH group showed preserved proliferative activity in contrast to the WH group. HSP70 expression decreased similarly in both groups. It is difficult to interpret these results, but the homozygous knockout (*hsp70.1*$^{-/-}$) status might have provided protection against reduced proliferative activity at the pre-translation level.

In bronchiolar epithelial cells, chronic hypoxia did not significantly increase apoptosis. The percentage of TUNEL-positive cell nuclei increased significantly in the KN group and the KH group compared with the WN group (P = 0.002 and P = 0.000, respectively). The genotype, oxygen conditioning and HSP70 expression could explain 32.6 % of the variance in apoptosis. Of these three variables, the genotype made the largest contribution (beta = 0.447) with statistical significance (P = 0.003). HSP70 is known to be a potent inhibitor of apoptosis, and these results indicate that HSP70 exerts an anti-apoptotic effect in bronchiolar epithelial cells and the reduced HSP70 expression in knockout (*hsp70.1*$^{-/-}$) mice results in an increase in apoptosis. The significant contribution to apoptosis by genotype, rather than HSP70 expression, may be due to the fact that HSP70 evaluated with the immunoblot method reflects the HSP70 expression at just one time-point, whereas genotype is likely to have a more sustained effect on HSP70 expression. The KH group showed a small increase in TUNEL-positive cell nuclei compared with the WH group, but statistical significance was not demonstrated. It is possible that hypoxia could inhibit the wild type of animals from displaying the anti-apoptotic mechanism fully, or that using more animals would make these differences statistically significant.

Despite recent advances, the anti-apoptotic mechanism of HSP70 remains controversial. It has been suggested that HSP70 exerts its protective effect downstream of caspase-3-like proteases (Jäättelä *et al.* 1998). In another study it was reported that HSP70 inhibits apoptosis downstream of cytochrome *c* release, but upstream of caspase-3 cleavage, and that the carboxyl-terminal region containing the peptide-binding domain is sufficient to inhibit caspase-3 activation (Li *et al.* 2000). HSP70 has also been reported to block apoptosis mediated by the caspase-independent death effector apoptosis-inducing factor (AIF), which is a mitochondrial intermembrane flavoprotein (Ravagnan *et al.* 2001).

In this study we investigated how epithelial cells of the airways respond to chronic hypoxia in terms of HSP70 expression, proliferation, and apoptosis. To our knowledge, this is the first study that has investigated how HSP70 expression in bronchiolar epithelial cells changes in response to chronic hypoxic stress. HSP70 expression and proliferation is significantly reduced by chronic hypoxia. The homozygous knockout (*hsp70.1*$^{-/-}$) status is a significant contributor to apoptosis. Therefore, HSP70 may exert anti-apoptotic effects in mouse bronchiolar epithelial cells.

References

BEN-SASSON SA, SHERMAN Y, GAVRIELI Y: Identification of dying cells – in situ staining. *Methods Cell Biol* **46**: 29-39, 1995.

DI CARLO A, WHITE N, GUO F, GARRETT P, LITOVITZ T: Chronic electromagnetic field exposure decreases HSP70 levels and lowers cytoprotection. *J Cell Biochem* **84**: 447-454, 2002.

DING XZ, SMALLRIDGE RC, GALLOWAY RJ, KIANG JG: Increases in HSF1 translocation and synthesis in human epidermoid A-431 cells: role of protein kinase C and [Ca^{2+}]i. *J Investig Med* **44**: 144-153, 1996.

FILIPOVIC D, GAVRILOVIC L, DRONJAK S, RADOJCIC MB: Brain glucocoid receptor and heat shock protein 70 levels in rats exposed to acute, chronic or combined stress. *Neuropsychobiology* **51**: 107-114, 2005.

JÄÄTTELÄ M: Overexpression of hsp70 confers tumorigenicity to mouse fibrosarcoma cells. *Int J Cancer* **60**: 689–693, 1995.

JÄÄTTELÄ M, WISSING D, BAUER PA, LI GC: Major heat shock protein hsp70 protects tumor cells from tumor necrosis factor cytotoxicity. *EMBO J* **11**: 3507-3512, 1992.

JÄÄTTELÄ M, WISSING D, KOKHOLM K, KALLUNKI T, EGEBLAD M: Hsp70 exerts its anti-apoptotic function downstream of caspase-3-like proteases. *EMBO J* **17**: 6124–6134, 1998.

JOHNSON D, OPPERMANN H, JACKSON J, LEVINSON W: Induction of four proteins in chick embryo cells by sodium arsenite. *J Biol Chem* **255**: 6975-6980, 1980.

KELLEY PM, SCHLESINGER MJ: The effect of amino acid analogues and heat shock on gene expression in chicken embryo fibroblasts. *Cell* **15**: 1277-1286, 1978.

KIANG JG, TSOKOS GC: Heat shock protein 70 kDa molecular biology, biochemistry, and physiology. *Pharmacol Ther* **80**: 183-201, 1998.

KIANG JG, WANG XD, DING XZ, GIST ID, SMALLRIDGE RC: Heat shock inhibits the hypoxia-induced effects on iodide uptake and signal transduction and enhances cell survival in rat thyroid FTRL-5 cells. *Thyroid* **6**: 475-483, 1996.

LEVINSON W, OPPERMANN H, JACKSON J: Transition series metals and sulfhydryl reagents induce the synthesis of four proteins in eukaryotic cells. *Biochem Biophys Acta* **606**: 170-180, 1980.

LI CY, LEE JS, KO YG, KIM JI, SEO JS: Heat shock protein 70 inhibits apoptosis downstream of cytochrome c release and upstream of caspase-3 activation. *J Biol Chem* **276**: 25665-25671, 2000.

MEYRICK B, REID L: Hypoxia and incorporation of ^{3}H-thymidine by cells of the rat pulmonary arteries and alveolar wall. *Am J Pathol* **96**: 51-70, 1979.

MOSSER DD, CARON AW, BOURGET L, DENIS-LAROSE C, MASSIE B: Role of human heat shock protein hsp70 in protection against stress-induced apoptosis. *Mol Cell Biol* **17**: 5317-5327, 1997.

NADZIEJKO CE, LOUD AV, KIKKAWA Y: Effect of alveolar hypoxia on pulmonary mast cells in vivo. *Am Rev Respir Dis* **140**: 743-748, 1989.

NOLLEN EA, BRUNSTING JF, ROELOFSEN H, WEBER LA, KAMPINGA HH: In vivo chaperone activity of heat shock protein 70 and thermotolerance. *Mol Cell Biol* **11**: 2069-2079, 1999.

POUYSSEGUR J, SHIU PC, PASTAN I: Induction of two-transformation-sensitive membrane polypeptides in normal fibroblasts by a block in glycoprotein synthesis or glucose deprivation. *Cell* **11**: 941-947, 1977.

RAVAGNAN L, GURBUXANI S, SUSIN SA, MAISSE C, DAUGAS E, ZAMZAMI N, MAK T, JÄÄTTELÄ M, PENNINGER JM, GARRIDO C, KROEMER G: Heat-shock protein 70 antagonizes apoptosis-inducing factor. *Nat Cell Biol* **3**: 839-843, 2001.

RILEY DJ, THAKKER-VARIA S, WILSON FJ, POIANI GJ, TOZZI CA: Role of proteolysis and apoptosis in regression of pulmonary vascular remodeling. *Physiol Res* **49**: 577-585, 2000.

SCHOLZEN T, GERDES J: The Ki-67 protein: from the known and the unknown. *J Cell Physiol* **182**: 311-322, 2000.

STORCH TG, TALLEY GD: Oxygen concentration regulates the proliferative response of human fibroblasts to serum and growth factors. *Exp Cell Res* **175**: 317-325, 1988.

SUZUKI K, WATANABE M: Modulation of cell growth and mutation induction by introduction of the expression vector of human HSP70 gene. *Exp Cell Res* **215**: 75-81, 1994.

WEI Y, ZHAO X, KARIYA Y, TESHIGAWARA K, UCHIDA A: Inhibition of proliferation and induction of apoptosis by abrogation of HSP70 expression in tumor cells. *Cancer Immunol Immunother* **40**: 73-78, 1995.

WEISS YG, MALOYAN A, TAZELAAR J, RAJ N, DEUTSCHMAN CS: Adenoviral transfer of HSP70 into pulmonary epithelium ameliorates experimental acute respiratory distress syndrome. *J Clin Invest* **110**: 801-806, 2002.

Reprint requests

June Dong Park, Department of Pediatrics, Seoul National University Hospital, 28 Yongon-dong Jongno-gu, Seoul, Korea 110-744. Fax: 822-762-3359. E-mail : jdparkmd@snu.ac.kr