Overexpressed heat shock protein 70 protects cells against DNA damage caused by ultraviolet C in a dose-dependent manner

Piye Niu,¹ Lin Liu,² Zhiyong Gong,¹ Hao Tan,¹ Feng Wang,¹ Jing Yuan,¹ Youmei Feng,² Qingyi Wei,¹ Robert M Tanguay,³ and Tangchun Wu¹

¹Institute of Occupational Medicine and The Ministry of Education Key Lab of Environment and Health, School of Public Health, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China

²Department of Biochemistry, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China

³Laboratory of Cell and Developmental Genetics, Department of Medicine, Faculty of Medicine, Pav. C.E. Marchand, Université Laval, Québec G1K 7P4, Canada

Abstract Heat shock protein 70 (Hsp70) comprises proteins that have been reported to protect cells, tissues, and organisms against damage from a wide variety of stressful stimuli; however, little is known about whether Hsp70 protects against DNA damage. In this study, we investigated the relationship between Hsp70 expression and the levels of ultraviolet C (UVC)–induced DNA damage in A549 cells with normal, inhibited, and overexpressed Hsp70 levels. Hsp70 expression was inhibited by treatment with quercetin or overexpressed by transfection of plasmids harboring the *hsp70* gene. The level of DNA damage was assessed by the comet assay. The results showed that the levels of DNA damage (shown as the percentage of comet cells) in A549 cells increased in all cells after exposure to an incident dose of 0, 10, 20, 40, and 80 J/m² whether Hsp70 was inhibited or overexpressed. This response was dose dependent: a protection against UVC-induced DNA damage in cells with overexpressed Hsp70 was observed at UVC dose 20 J/m² with a maximum at 40 J/m² when compared with cells with normal Hsp70 levels and in quercetin-treated cells. This differential protection disappeared at 80 J/m². These results suggest that overexpressed Hsp70 might play a role in protecting A549 cells from DNA damage caused by UVC irradiation, with a threshold of protection from at UVC irradiation-induced DNA damage by Hsp70. The detailed mechanism how Hsp70 is involved in DNA damage and possible DNA repair warrants further investigation.

INTRODUCTION

Heat shock protein 70 (Hsp70) comprises highly conserved proteins induced in cells on exposure to supra-optimal temperatures or many other forms of stress, such as ultraviolet irradiation, oxidizing agents, and organic solvents, which are very common in working and living environments (Bonaventura et al 2005; Mayer and Bukau 2005). Induced Hsp70 protects cells, tissues, and organisms against damage from a wide variety of stressful stimuli (Hightower 1991; Wu et al 1996, 2001). Under

nonstressful conditions, Hsp70, a cytoplasmic protein, has multiple housekeeping functions, such as folding and translocating newly synthesized proteins, activating specific regulatory proteins, replicating proteins and kinases, and degrading proteins (Helmbrecht et al 2000; Jolly and Morimoto 2000). Hsp70 can translocate to the nucleus and accumulate there under heat shock or in other harmful conditions (Szekely et al 1995; Chughtai et al 2001; Lepock et al 2001). Whether Hsp70 in the nucleus can protect against DNA damage is still unknown.

Few studies exist on a possible relationship between Hsp70 levels and DNA damage. Investigations on the effects of the heavy metal cadmium on a marine sponge suggested that DNA damage was responsible for Hsp70 induction (Schroder et al 1999). Galvano et al (2002) re-

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Correspondence to: Professor Tangchun Wu, Tel: 86 27 83692347; Fax: 86 27 83692560; E-mail: wut@mails.tjmu.edu.cn.

ported that fumonisin B1 induced the expression of Hsp70 in an early response to cellular stress and that Hsp70 expression was not strictly related to the level of DNA damage. A few investigations suggest that Hsps might be involved DNA repair; for example, Zou et al (1998) found that DnaK (Hsp70) participated in nucleotide excision repair by maintaining repair proteins in their properly folded state in *Escherichia coli*. Ciavarrà et al (1994) reported that nuclear type I topoisomerase complexes with Hsc70 might limit heat-induced protein damage, accelerate restoration of protein function in an ATP-independent reaction, or both. Some studies showed that Hsp70 can improve base excision repair by interacting with human apurinic/apyrimidinic endonuclease and by stimulating single-strand gap-filling by DNA polymerase beta (Mendez et al 2000, 2003; Kenny et al 2001; Bases 2005). In a previous study, we found a significantly negative correlation between Hsp70 levels and the level of DNA damage as measured by the comet assay in workers exposed to coke oven emissions (Xiao et al 2002). However, the protective effects might have been confounded by factors of unknown physical and genetic conditions. Therefore, we investigated the possible role of Hsp70 in protection against DNA damage in A549 cells by either inhibiting or overexpressing Hsp70 with the use of ultraviolet C (UVC) irradiation. UVC-induced DNA damage was measured by the comet assay (Herrlich et al 1992; Tyrrell 1996). We tested the hypothesis that the levels of UVC-induced DNA damage were correlated with levels of Hsp70 expression in A549 cells under conditions in which this Hsp is inhibited or overexpressed.

MATERIALS AND METHODS

Cell culture

The A549 cell line used in this study was purchased from the China Center of Type Culture Collection in Wuhan, China. The A549 cells, a human lung adenocarcinoma cell line, were grown in Dulbecco modified Eagle minimal essential medium (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal calf serum (Sigma, St Louis, MO, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin and incubated in a humidified atmosphere containing 5% CO₂ and 95% air at 37°C. The cell density was adjusted to less than 1×10^6 cells/mL. All experiments were performed with cells in the logarithmic stage of growth. Cells were counted with an electronic counter (Casy-1, Schärfe Systems, Reutlingen, Germany).

Pretreatment with quercetin

Cells were seeded in triplicate at 1×10^6 cells per well in 6-well tissue culture plates (Corning, New York, NY,

USA) for 12 hours and then treated with 0.1% dimethyl sulfoxide (DMSO) alone as solvent control or with different concentrations of quercetin (Shanghai Biochemical Reagent Factory, Shanghai, P.R. China) in DMSO at 50, 100, 150, or 200 µM at 37°C for 6 hours. After treatment, the culture medium was replaced with fresh culture medium and the cells were then treated by heat shock at 41°C for 1 hour followed by 2 hours of recovery at 37°C. Control cells were cultured at 37°C.

Gene transfection

The A549 cells were transfected with pcDNA3.0/*hsp70* with Lipofectamine 2000 Reagent (Life Technologies, Inc, Gaithersburg, MD, USA). After transfection, transfected cells were selected according to a modification of a previously described method (Jäättelä et al 1992). Positive clones of A549 cells were selected with antibiotic G418 (800 µg/mL; GIBCO) for 2 weeks. The clonal populations were expanded and analyzed for the expression of Hsp70 by Western blot analysis as previously described (Jäättelä et al 1992). G418 (400 mg/mL) was used to maintain the clones in culture. A control population of A549 cells was also transfected with a pcDNA3.0 plasmid containing the neomycin-resistance gene but not *hsp70* cDNA.

Determination of Hsp70 expression

The Western blot assay was performed as previously described (Wu et al 1998). Briefly, the treated cells were harvested and incubated on ice for 15 minutes in a lysis buffer of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 100 µg/mL phenyl methylsulfonyl fluoride, 1 µg/mL aprotinin, and 1% Triton X-100. Cell debris was removed by centrifugation at 10 000 rpm and 4°C for 10 minutes. The protein concentration of each cell lysate was determined with a Bio-Rad (Hercules, CA, USA) protein assay kit. To each tube, an equivalent volume of 2× sodium dodecyl sulfate (SDS) loading buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerine, 10% β-mercaptoethanol, and 0.2% bromophenol blue) was added and mixed again. The mixtures were then denatured at 95°C for 10 minutes, and about 10 µg of the protein mixture was loaded and separated in each well on 10% SDS-polyacrylamide electrophoresis gels. After separation for about 80 minutes, the proteins were transblotted onto nitrocellulose membranes (Bio-Rad), and the membranes were saturated and blocked with 5% fat-free milk at 37°C for 1 hour. Membranes were probed with rabbit polyclonal anti-Hsp70 (#799; Tanguay et al 1993), mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Kangcheng, Shanghai, P.R. China) antibodies (1:5000 to 1:10 000 dilution) and then with horseradish peroxidase-conjugated secondary immunoglobulin G (IgG, Kang-

cheng). The membranes were then treated with an enhanced chemiluminescence reagent (Amersham, Piscataway, NJ), and the signals were detected by exposure of the membranes to X-ray films (Kodak, Rochester, NY, USA). The relative signal intensity was quantified by densitometry with Gel pro3.0 image software (Media Cybernetics, Silver Spring, MD, USA) on an IBM-compatible personal computer. All experiments were independently performed 3 times.

UVC irradiation

A549 cells (control), A549 cells pretreated with DMSO (A549/DMSO) or with quercetin (A549/quercetin), and A549 cells transfected with pcDNA3.0 (A549/pcDNA) or pcDNA3.0 containing *hsp70* gene (A549/*hsp70*) were put in 6-well tissue culture plates in triplicate at 1×10^6 cells per well and cultured for 12 hours. For UVC irradiation, cells were washed with warm phosphate-buffered saline and irradiated at various doses with a 254-nm UV lamp (Shanghai Experiment Reagent Co Ltd, Shanghai, P.R. China) precalibrated with an ultraviolet radiometer (Photoelectric Instrument Factory of Beijing Normal University, Beijing, P.R. China). After irradiation, fresh culture medium was added to cells, which were cultured at 37°C for 15 minutes for recovery (Duann et al 1999).

Detection of DNA damage by the comet assay

The alkaline comet assay was performed as previously described (Singh et al 1988) with some modification (Moeneef et al 2003). Briefly, a total of 100 μ L from the cell suspension (1×10^5 cells) was embedded in 1% agarose, spread over a frosted slide, and immediately covered with a cover glass to make a microgel on the slide. Slides were placed immediately in cold lysis buffer (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris, pH 10, and 1% Triton X-100, 4°C) for a minimum of 1 hour. After lysis, the slides were drained and placed in a horizontal gel electrophoresis tank surrounded by ice and filled with fresh cold electrophoresis buffer (300 mM NaOH, 1 mM Na₂EDTA, pH 13) to a level of approximately 0.25 cm above the slides. Slides were kept in the high pH buffer for 20 minutes to allow DNA unwinding. Electrophoresis was then carried out for 20 minutes at 25 V and 300 mA. The slides were then drained and flooded slowly with 3 changes of neutralization buffer (0.4 M Tris, pH 7.5) for 5 minutes each and then stained with 50 μ L of ethidium bromide (20 μ g/L) and covered with coverslips. To prevent additional DNA damage from visible light, all the steps described above were conducted under a dimmed light.

A total of 50 randomly selected cells per slide were analyzed (ie, 150 cells per specific treatment; Price et al 2000). Image analysis was performed at 200 $\times$ magnifi-

cation with a fluorescence microscope (Olympus B-60F5, Japan) attached to a digital camera (Olympus, Tokyo, Japan) equipped with an excitation filter of 549 nm, a barrier filter of 590 nm, and a 100-W mercury lamp. The images were analyzed by the IBM-compatible personal computer-based image analysis system IMI 1.0 (Zhu et al 2001). The comet tail moments are positively correlated with the level of DNA breakage in a cell (Singh et al 1988). The mean values of the Olive tail moment (OTM) in a particular sample were taken as an index of DNA damage for this sample. OTM is defined as the fraction of tail DNA multiplied by the distance between the profile centers of gravity for DNA in the head and tail (Olive et al 1992). OTM was measured from 3 independent experiments, each containing triplicate measures and presented as the mean $\pm$ standard error of mean (SEM). Cells with undamaged DNA were verified as described previously (Wollowski et al 1999). In brief, tail DNA of less than 5% was considered undamaged DNA. The percentage of comet cells was calculated by the formula: Comet cells (%) = (comet cells/total cells) $\times$ 100.

Statistical analysis

Statistical analyses were performed by 1-way analysis of variance followed by the least significant difference test. In all tests, differences were considered significant at $P < 0.05$ and very significant at $P < 0.01$. All data analyses were carried out with the use of statistical analysis software SPSS12.0 for Windows (SPSS Inc, Chicago, IL, USA).

RESULTS

Inhibition of Hsp70 expression by quercetin

As shown in Figure 1, Hsp70 increased markedly after heat shock at 41°C for 1 hour. The increase in Hsp70 was inhibited by the treatment of cells with quercetin (50–200 μ mol) for 6 h before heat shock at 41°C for 1 hour. Compared with the control group (also at 41°C for 1 hour), Hsp70 levels decreased significantly in the A549 cells exposed to 100, 150, and 200 μ M quercetin ($P < 0.01$ for all groups). The Hsp70 level was lower at 150 μ M ($P < 0.05$) than at 100 μ M, but no significant difference was found between the 150 and 200 μ M groups ($P > 0.05$). Cell viability was more than 91% at concentrations of 50, 100, and 150 μ M quercetin but less than 85% at a concentration of 200 μ M quercetin. The concentration of 200 μ M quercetin could have induced apoptosis. Therefore, 150 μ M quercetin was used to inhibit the expression of Hsp70 expression of all A549 cells in subsequent experiments.

Fig 1. (A) Relative expression of Hsp70 in A549 cells treated by increasing quercetin (50, 100, 150, 200 μ M) at 37°C for 6 hours and then heat shocking (41°C, 1 hour) followed by for 2 hours of recovery at 37°C. 0 μ M is a positive control. DMSO (0.1%) was performed to treat cells as a solvent control because quercetin was dissolved in DMSO. A549 cells cultured at 37°C were used as a normal control. Protein (10 μ g) was used for immunoblot analysis of Hsp70 and GAPDH. Experiments were performed 3 times. (B) Hsp70 levels were quantitated by densitometric analysis of the autoradiography from (A). Vertical bars represent mean $\pm$ SEM of separate determinations, $n = 3$. Significantly different from the 0 μ M treatment of quercetin at ** $P < 0.01$.

Hsp70 levels in different groups of A549 cells

Figure 2 shows the levels of Hsp70 expression of different groups of A549 cells. There was a significant increase in Hsp70 levels when cells were transfected with the Hsp70-expressing gene (A549/hsp70), whereas a significant decrease in Hsp70 levels was observed in quercetin-treated cells (A549/quercetin; $P < 0.01$). However, Hsp70 levels did not change significantly in A549/DMSO and A549/pcDNA ($P > 0.05$) compared with control A549 cells.

DNA damage in A549 cells with different Hsp70 levels

To assess DNA damage, we used the comet assay. Figure 3 shows examples of the nucleoid figures observed. Nuclei from control cells had a head (nucleoid core) with a minimum amount of DNA migrating into the tail region (Fig 3A). Nuclei of A549 cells exposed to UVC irradiation had a head (reduced nucleoid core) with different DNA fragments migrating into the tail region as a result of strand breaks: small migration (Fig 3B), medium migration (Fig 3C), and major migration (Fig 3D). DNA frag-

Fig 2. (A) Hsp70 expression in A549 cells transfected by the *hsp70* gene (A549/hsp70) or treated by 150 μ M quercetin (A549/quercetin). Cells transfected by pcDNA3.0 vector (A549/pcDNA) were used as a control for A549/hsp70. Cells treated by 0.1% DMSO (A549/DMSO) were a solvent control of A549/quercetin. Protein (10 μ g) was used for immunoblot analysis of Hsp70 and GAPDH. Experiments were performed 3 times. (B) Hsp70 levels were quantitated by densitometric analysis of the autoradiography from (A). Vertical bars represent mean $\pm$ SEM of separate determinations, $n = 3$. Significantly different from the corresponding control at ** $P < 0.01$: A549/hsp70 vs A549/pcDNA and A549/quercetin vs A549/DMSO. No significant differences were found in A549/pcDNA vs A549 or A549/DMSO vs A549 ($P > 0.05$).

ments in the tails of comet cells increased with exposure to increasing UVC doses (10, 20, 40, 80 J/m²).

A549 cells in which Hsp70 was inhibited by quercetin or overexpressed by transfection with the Hsp70 plasmid were treated with increasing exposure to 10, 20, 40, and 80 J/m² UV irradiation. Figure 4 shows that the levels of DNA damage (ie, the percentage of comet cells) increased in control A549 cells A549/quercetin, and A549/hsp70 cells exposed to 10, 20, 40, and 80 J/m². No protection against DNA damage was obvious in cells with overexpressed Hsp70 when exposed to either low (10 J/m²) or high (80 J/m²) UVC compared with the control A549 cells or with A549 cells with inhibited Hsp70. The highest level of DNA damage was observed at 40 J/m² in the A549 cells with inhibited Hsp70, pointing to a role for Hsp70 in protection against DNA damage. Under our experimental conditions, the protection of Hsp70 against DNA damage started at 20 J/m² UVC irradiation and was maximum at 40 J/m² UVC irradiation compared with the control A549 cells and A549 cells with inhibited Hsp70. Un-

Fig 3. DNA damage of A549 cells was detected by comet assay. Nuclei from control cells consist of a head (nucleoid core) with a minimum amount of DNA migrating into the tail region (A). Nuclei of the A549 cells exposed to UVC irradiation consist of a head (nucleoid core) with DNA migrating into the tail region as a result of strand breaks: a little migration (B), medium migration (C), and major migration (D).

der the irradiation of 40 J/m² UVC, the percentage of comet cells in the A549/*hsp70*, control A549, and A549/ quercetin cells were 30.3%, 43.3%, and 53%, respectively. Comet cells were not significantly different among the groups of the control A549, A549/DMSO, and A549/ pcDNA treated by various doses of UVC irradiation (data not shown). The results of OTM values of DNA damage in the A549/quercetin, control A549, and A549/*hsp* cells at exposure to 40 J/m² UV irradiation are listed in Table 1. There was a significant decrease in OTM values in the A549/*hsp70* cells ($P < 0.01$) and a significant increase in the A549/quercetin cells ($P < 0.01$) compared with control A549 cells. OTM values were not significantly different in the A549/DMSO or A549/pcDNA cells compared with control A549 cells.

DISCUSSION

Hsp70 can be induced by a variety of adverse environmental and pathophysiological conditions, including hyperthermia, sulfhydryl reagents, or transition metal ions (Lindquist 1986; Lindquist and Craig 1988; Macario and Conway de Macario 2005). Overexpression of Hsp70 by transfection of *hsp70* cDNA has been reported to enhance myocardial tolerance to ischemia-reperfusion injury in rats (Suzuki et al 1997, 2002; Marber et al 1995) and in mice (Plumier et al 1995, 1997; Trost et al 1998). Furthermore, direct gene delivery of *hsp70* in vivo reduces the severity of ischemic injury in the rabbit heart (Okubo et al 2001). Quercetin, a flavone group compound, has been shown to block binding of Hsf1 to the heat shock element,

Fig 4. A549 cells with different Hsp70 levels were treated with increasing UV irradiation (0, 10, 20, 40, and 80 J/m²). Cells were irradiated at 0, 15, 30, 60, and 120 seconds. The cells with undamaged DNA were verified as described previously (Wollowski et al 1999). The percentage of comet cells was calculated by the formula: Comet cells (%) = (comet cells/total cells) × 100. No significant difference was found among A549, A549/pcDNA, and A549/DMSO cells (figures are not shown). The percentage of comet cells increased with increasing UV irradiation dosages and added up to 100% at the UV irradiation dose of 80 J/m² in all of the cells with different Hsp70 expression. The error bar indicates standard error of the mean from the 3 separate triplicate experiments.

thereby blocking the expression of Hsp70 (Hosokawa et al 1990, 1992; Kantengwa and Polla 1991; Koishi et al 1992; Elia and Santoro 1994; Elia et al 1996). Thus quercetin was found to inhibit Hsp70 synthesis for 3–6 hours in a carcinoma cell line after treatment with prostaglandin A1 (Elia et al 1996; Wei et al 1996).

The involvement of heat shock proteins in cellular resistance to the deleterious effects of UV irradiation is still controversial. Heat shock proteins can play roles in cellular defense mechanisms because cells preconditioned by heat shock were reported to be resistant to UVB (principally 290–320 nm) irradiation (Maytin et al 1993, 1994; Trautinger et al 1995). Overexpression of Hsp27 did not confer resistance to UVB (40–640 kJ/m²) irradiation in human squamous cell carcinoma cell lines (Kindas-Mugge et al 1996; Trautinger et al 1997). Wano et al (2004) investigated the removal capacities of damaged DNA in RSa and AP⁺-1 cells irradiated by 10 J/m² UVC. Up-regulation of Hsp27 could moderately elevate the removal capacities, suggesting that Hsp27 was possibly involved in the UVC resistance of human cells via nucleotide excision repair (Wano et al 2004). However as yet, no direct evidence for a protective role of Hsp70 against UV irradiation has been shown.

Our results show that UVC irradiation induced dose-dependent DNA damage in A549 cells and that Hsp70 seemed to confer some protection against DNA damage at a dose of 40 J/m² UVC irradiation. At this dose, inhibition of Hsps by quercetin increased DNA damage,

TABLE 1 OTM values of DNA damage caused by 40 J/m² ultraviolet C irradiation (mean $\pm$ SEM, $n = 3$)

Group	OTM		P values ^a	
A549	18.83 $\pm$ 0.41	Reference	0.872	0.655
A549/pcDNA	18.67 $\pm$ 0.81	0.655	Reference	0.773
A549/DMSO	18.38 $\pm$ 0.73	0.872	0.773	Reference
A549/hsp70	14.31 $\pm$ 1.60	<0.001	0.001	0.002
A549/quercetin	23.95 $\pm$ 1.75	0.001	<0.001	<0.001

OTM, olive tail moment (fraction of tail DNA multiplied by the distance between the profile centers of gravity for DNA in the head and tail); A549, normal control; A549/pcDNA, control for A549/hsp70; A549/DMSO, control for A549/quercetin.

^a One-way analysis of variance followed by least significant difference test.

whereas the overexpression of Hsp70 decreased it. At UVC irradiation doses higher than 40 J/m², protection against DNA damage was diminished, partly because Hsp70 expression levels became down-regulated, whereas the decreased level of DNA damage in the A549/quercetin cells is likely an apoptotic effect of the excess of DNA damage. These results suggest a threshold of protection of Hsp70 against DNA damage induced by UVC irradiation. It is possible that the absence of protection of Hsp70 against DNA damage at low doses of UVC might result from a limitation of the comet assay in its sensitivity for detecting low levels of DNA damage.

Various functions have been attributed to Hsp70 not only in protein folding, protein translocation through membranes, and protein degradation but also in the translation machinery in normal and stressed states (Nelson et al 1992), which could be very important for the damage and denaturation of cells caused by UVC. Hsp70 also shares ATPase activities that are thought to be necessary for the release of associated peptides (Lewis and Pelham 1985; Mayer and Bukau 2005; Shomura et al 2005). Hsp70 can inhibit apoptosis by modulating the intracellular p53 function (Chen et al 1999; Lee et al 2001; Park and Nakamura 2005). The p53 functions as a transcription factor regulating expression of genes involved in DNA damage response pathways that affect apoptosis, DNA repair, and cell cycle regulation (Balint and Vousden 2001). Taking this into account, we can speculate that Hsp70 could be involved in the signaling of protein synthesis in the p53–DNA damage pathway that modulates DNA repair. In addition, Hsp70 might act at the DNA repair level because this Hsp has been shown to interact with the base excision repair enzymes (Mendez et al 2000; Kenny et al 2001) and stimulate DNA polymerase beta gap-filling activity (Mendez et al 2003).

The data presented here show that exposure of A549 cells to UV irradiation results in DNA damage and that an increase in Hsp70 expression can in turn protect cells from DNA damage in a dose-dependent manner. This suggests that Hsp70 might play a role in protecting A549 cells against UVC-induced DNA damage in a limited range of exposure. However, the detailed mechanisms of how Hsp70 is involved in the activities of the DNA dam-

age and repair response pathway warrants further investigation.

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