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A Priming Heat Treatment Can Induce the Development of Heat- and Radio-resistance *via* HSPs, Regardless of *p53*-gene Status

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Abstract : It has been suggested that inducible heat shock proteins (HSPs) may function in multiple roles in cytoprotection. However, recent reports have shown that nitric oxide (NO) radicals are an initiator of heat- and radio-resistance, and act through the activation of the human homolog of MDM2 (HDM2), the depression of p53 accumulation, and the induction of NO synthase (iNOS, or alternatively, NOS2) which is observed following a priming irradiation. The aim of this work was to acquire additional information on the roles of p53, HDM2, iNOS, NO radicals, and HSPs on the development of heat- and radio-resistance as following a priming heat treatment. Wild-type (wt) *p53* and mutated (m) *p53* cells were used. These cells were derived from the H1299 human lung cancer cell line in which *p53* is deleted. Cellular sensitivities were determined with a colony-forming assay. In both pre-heated wt*p53* cells and in pre-heated mp53 cells, the induction of heat- and radio-resistance was observed in the absence of KNK437 (an inhibitor of HSPs), and in the presence of RITA (an inhibitor of p53-HDM2 interactions), aminoguanidine (an iNOS inhibitor) or c-PTIO (an NO radical scavenger). These findings suggest that following a priming heat treatment, HSPs contribute to heat- and radio-resistance.

Key Words : heat-resistance, radio-resistance, HSP, p53, NO

Introduction

Many environmental agents which are present at low concentrations are able to induce an increased resistance to subsequent lethal insults. Exposure of cells to a transient, non-lethal elevation in temperature results in the activation of cellular stress responses and induces heat-resistance in cells[1]. Moreover, it was reported that mild hyperthermia can induce an adaptation or resistance to cytogenetic damage caused by subsequent X-irradiation[2]. Although heat-resistance is associated with the synthesis and cellular accumulation of a family of highly conserved proteins referred to as heat shock proteins (HSPs)[3-5], little is known about their mechanism of action, or about any association with observed radio-resistance following a priming heat treatment.

It has been reported that conditioning exposures of X-radiation at low doses and at low dose-rates

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reduce heat- and radiation-induced tumor suppressor gene *p53*-dependent apoptosis in cultured cells[6]. A conditioning radiation exposure has also been reported to suppress p53 function[7]. These findings led to a proposal suggesting that this repressed *p53*-dependent response is one of the mechanisms likely to be involved in the radioadaptive response[8]. A possible model of signaling pathways has been proposed to describe the induction of heat- and radio-resistance by a low dose pre-irradiation, and includes the following steps: (a) a priming irradiation activates the human homolog of MDM2 (HDM2) during the interval between the priming and challenging irradiation or heat-treatment; (b) HDM2 leads to the degradation of p53 through ubiquitination; (c) the decrease in p53 relaxes a depression of inducible nitric oxide (NO) synthase (iNOS, or alternatively, NOS2) induction; (d) the challenging irradiation or (e) heat shock induces an accumulation of iNOS; (f) iNOS generates NO radicals; (g) NO radicals lead to an induction of heat- and radio-resistance[9–11].

In studying the induction of heat- and radio-resistance produced by a priming heat treatment the work described here is focused on the role of p53, which is known to play a key role in protecting the genome[12], and in the activation of p53 in response to heat-treatment[13]. To learn if HDM2, NO radicals, and HSPs contribute to the induction of heat- and radio-resistance after a priming heat treatment, the effects of specific inhibitors were studied.

Materials and methods

Chemicals

5, 5'-(2, 5-Furanidiyl) bis-2-thiophenemethanol (RITA), an inhibitor of p53-HDM2 interactions[14] was purchased from Tocris Cookson Ltd. (Avonmouth, U.K.). Aminoguanidine, an inhibitor of iNOS[15] was purchased from Sigma Aldrich Inc. (St Louis MO, USA). 2-(4-Carboxyphenyl)-4, 4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO), an NO radical scavenger[16] was purchased from Doujin Chemical Co. (Tokyo, Japan). KNK437 (*N*-formyl-3, 4-methylenedioxy-γ-butyrolactam), an inhibitor of HSPs[17] was provided by the Kaneka Co. (Osaka, Japan). Giemsa solutions were purchased from Merck Ltd. (Tokyo, Japan).

Cells

Human H1299 non-small cell lung cancer cells with a deleted *p53* gene (provided by Dr. Moshe Oren, Weizmann Institute of Science, Rehovot, Israel) were stably transfected with either a wild-type (wt) *p53* gene or a mutated (m) *p53* gene (in which codon 248, is altered to code for Trp [TGG]) rather than Arg [CGG]). The cell lines with a wt*p53* or a m*p53* gene are designated H1299/wt*p53* or H1299/m*p53* cells, respectively[18]. These resulting H1299/wt*p53* and H1299/m*p53* cell lines were kindly provided by Dr. Matsumoto (University of Fukui). H1299/m*p53* cells have lost p53 functions such as the induction of apoptosis and *p53*-regulated gene products after exposure to X-rays[19]. All cells were cultured in Dulbecco's modified Eagle's medium (MP Biomedicals Inc., Illkirch, France) containing 10% (v/v) fetal bovine serum (MP Biomedicals Inc.), 20 μmol/ml *2*-[*4*-(*2*-hydroxyethyl)-*1*-piperazinyl] ethanesulfonic acid (Nacalai Tesque, Kyoto, Japan), 50 units/ml penicillin (Meiji Seika Kaisha Ltd., Tokyo, Japan), 50 μg/ml streptomycin (Meiji Seika Kaisha Ltd.), and 50 μg/ml kanamycin (Nacalai Tesque) (DMEM-10). The doubling time of these cell lines was about 24 h. Exponentially growing cells which were grown to a density of about 80% of confluency were used for each experiment, and were cultured

at 37°C in a conventional humidified CO_2 incubator.

Heat-treatment

For heat treatments, cell culture flasks containing H1299/wt*p53* or H1299/m*p53* cells were immersed in a water bath (Thermominder EX ; Taitec Co., Ltd., Koshigaya, Japan) maintained at 44±0.1°C. Priming heat conditions were 44°C for 5 min for both cell lines. For the challenging heat treatment (resulting in a survival rate of about 20%), H1299/wt*p53* and H1299/m*p53* cells were heated at 44°C for 45 min and 60 min, respectively, because the heat-sensitivity of H1299/wt*p53* cells was about 1.3-fold higher than that of the H1299/m*p53*[11] cells. After heat treatments, cells were cooled down immediately, and then incubated at 37°C in a humidified CO_2 incubator.

X-Irradiation

X-ray (1.0 Gy/min, 20 mA) exposures were delivered with a 150-kVp X-ray generator (Model MBR-1520R, Hitachi, Ltd., Tokyo, Japan). H1299/wt*p53* and H1299/m*p53* cells were irradiated with 6 and 8 Gy, respectively (these doses resulted in approximately a 20% survival rate) because the radio-sensitivity of H1299/wt*p53* cells to X-rays was about 1.36 times higher than that of H1299/m*p53* cells[11,20].

Treatment with RITA, AG or c-PTIO

RITA, AG or c-PTIO were treated as previously described[9−11]. Seven hours prior to the challenging heat-treatment or irradiation, cells were washed twice with DMEM-10 and then incubated in DMEM-10 containing 10 μM RITA (dissolved in dimethyl sulfoxide, DMSO) or 50 μM AG (dissolved in PBS). Immediately prior to the challenging heat-treatment or irradiation, cells were washed twice with DMEM-10 and then placed in DMEM-10 containing 10 μM c-PTIO (dissolved in PBS). The final concentration of each solvent was 0.02%. Cells were then incubated at 37°C in a conventional humidified CO_2 incubator, with no subsequent medium changes, to permit the formation of colonies.

Treatment with KNK437

KNK437 was treated as previously described[5,21]. KNK437 was dissolved in DMSO and added to the culture medium at a final concentration of 0 or 300 μM at 7 h prior to the challenging heat-treatment or irradiation. For cell colony forming assays, the medium containing KNK437 was exchanged for KNK437-free medium 10 h after the challenging heat-treatment or irradiation.

Survival curves

Exponentially growing cells were treated at 6 h after plating in 25 cm^2 culture flasks. The surviving cell fraction was determined using colony-forming assays. Three replicate flasks were used per experiment, and two or more independent experiments were performed for each survival point. Colonies were fixed with methanol and stained with a 2% Giemsa solution. Microscopic colonies containing more than approximately 50 cells were counted as having arisen from single surviving cells. The error bars indicate standard deviations.

Statistical analysis

Significance levels were calculated using the Student's t-test. Values of $P<0.05$ were considered statistically significant.

Results

Induction of heat- and radio-resistance by a priming heat treatment

The effect of the time interval (0-18 h) between the priming heat treatment and the challenging heat treatment or irradiation on the induction of heat- or radio-resistance was examined in human lung cancer H1299/wt*p53* and H1299/m*p53* cells (Fig. 1). Induction of heat-resistance was evident at time intervals of over 3 h after the priming heat treatment in both cell lines (Fig. 1*a*). The protective effects of the priming heat treatment continued up to 18 h after the priming heat treatment (Fig. 1*a*). Induction of heat- and radio-resistance reached its maximum effect at 6 h after the priming heat treatment in both cell lines (Figs. 1*a* and *b*).

These data also show that thermal enhancement of cellular radio-sensitivity in response to X-rays was seen in H1299/wt*p53* cells which were irradiated with X-rays immediately after the priming heat treatment, but not in H1299/m*p53* cells (Fig. 1*b*).

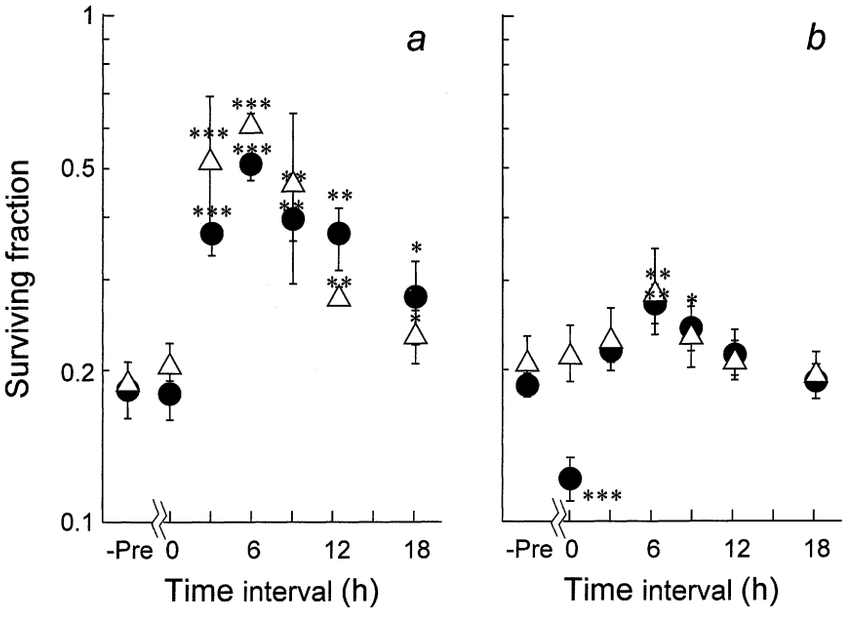

Fig. 1. Effect of time intervals (between 0 and 18 h) on the induction of heat- and radio-resistance after a priming heat treatment. The closed circles, (●) represent wt*p53* cells (H1299/wt*p53*), and the open triangles (△) represent m*p53* cells (H1299/m*p53*). Panel ***a***: H1299/wt*p53* and H1299/m*p53* cells were exposed to a challenging heat by being heated at 44°C (for 45 and 60 min, respectively) at various time points (0 to 18 h) after a priming heat treatment at 44°C for 5 min. Panel ***b***: H1299/wt*p53* and H1299/m*p53* cells were exposed to a challenging irradiation dose (6 and 8 Gy, respectively) at various time points (0 to 18 h) after a priming heat treatment at 44°C for 5 min. Asterisks (*, ** and ***) indicate statistically significant differences ($P<0.05$, 0.01 and 0.001, respectively) according to the Student's *t*-test.

The effect of an HDM2 inhibitor on the induction of heat- and radio-resistance by a priming heat treatment

HDM2 serves as an ubiquitin ligase and targets p53 for degradation. Thus the effect of RITA (a specific inhibitor of the interaction between p53 and HDM2, and present at a concentration of 10 μM) on the induction of heat- and radio-resistance was of interest. It can be seen that the induction of heat- and radio-resistance by a priming heat treatment was not suppressed by the addition of RITA to the culture medium in either cell line (Fig. 2).

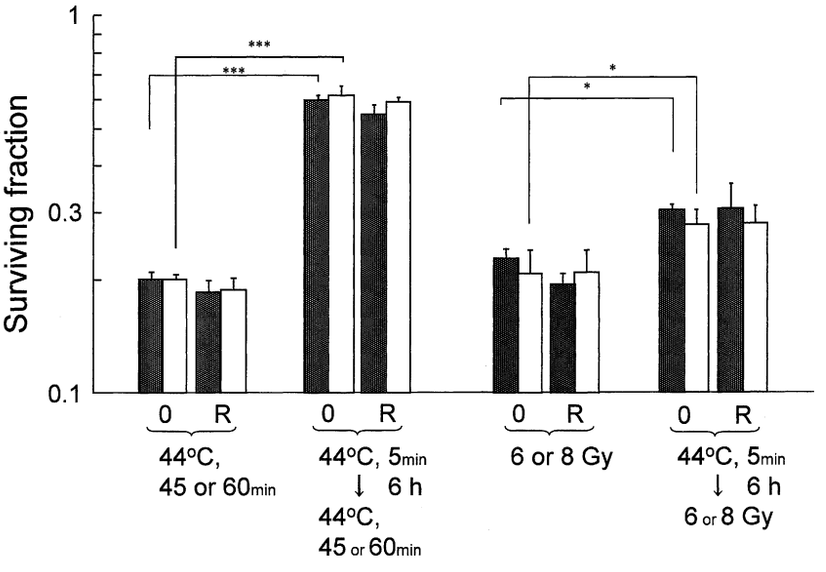

Fig. 2. Effect of an HDM2 inhibitor pretreatment on the induction of heat- and radio-resistance. Cells were treated with heat shock or irradiation at 6 h after a priming heat treatment at 44°C for 5 min. H1299/wt*p53* cells (closed columns) and H1299/m*p53* cells (open columns) were heated at 44°C for 45 and 60 min or irradiated with 6 and 8 Gy, respectively. Labels: **0**, no inhibitor; **R**, 10 μM RITA (an inhibitor of p53-HDM2 interactions). RITA was added 7 h before the start to challenging heat-treatment or irradiation. Asterisks (* and **) indicate statistically significant differences ($P<0.05$ and 0.01, respectively) using the Student's *t*-test.

The effect of an iNOS inhibitor on the induction of heat- and radio-resistance by a priming heat treatment

If aminoguanidine (a specific inhibitor of iNOS, present at a concentration of 50 μM) was added to the culture medium 7 h before a challenging heat-treatment or challenging irradiation, the induction of heat- and radio-resistance by a priming heat treatment was not significantly affected in either cell line (Fig. 3).

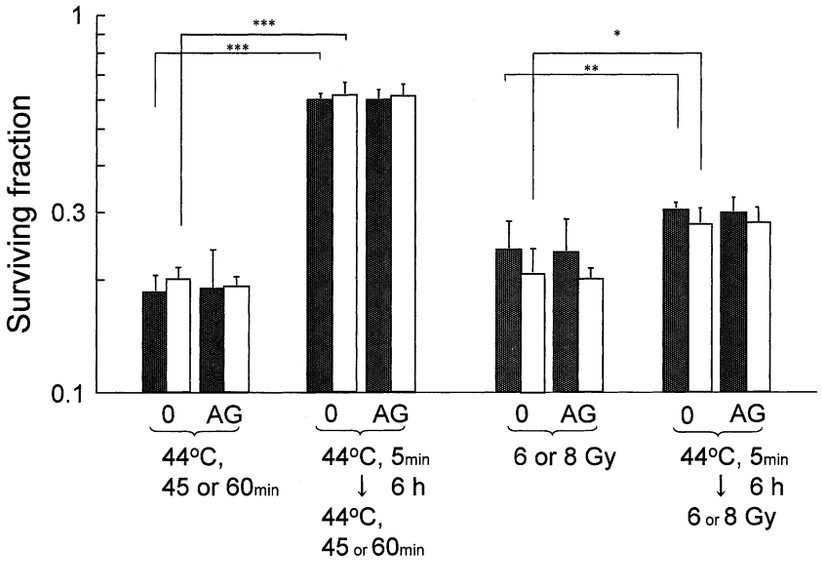

Fig. 3. Effect of an iNOS inhibitor pretreatment on the induction of heat- and radio-resistance. Cells were treated with heat shock or irradiation at 6 h after a priming heat treatment at 44°C for 5 min. H1299/wt*p53* cells (closed columns) and H1299/m*p53* cells (open columns) were heated at 44°C for 45 and 60 min or irradiated with 6 and 8 Gy, respectively. Labels: **0**, no inhibitor; **AG**, 50 μM aminoguanidine (an iNOS inhibitor). Aminoguanidine was added 7 h before the start to challenging heat-treatment or irradiation. Asterisks (*, ** and ***) indicate statistically significant differences ($P<0.05$, 0.01 and 0.001, respectively) according to the Student's *t*-test.

The effect of a NO radical scavenger on the induction of heat- and radio-resistance by a priming heat treatment

Fig. 4 shows the surviving fractions of H1299/wt*p53* cells and of H1299/m*p53* cells in the presence and absence of c-PTIO (a specific scavenger of NO radicals, present at a concentration of 10 μM in the medium). The induction of heat- and radio-resistance by a priming heat treatment was not suppressed by the addition of c-PTIO to the culture medium just before the challenging heat-treatment or irradiation in either cell line (Fig. 4).

The effect of a HSP inhibitor on the induction of heat- and radio-resistance induced by a priming heat treatment

If KNK437 (a specific inhibitor of HSPs) was present in the medium at a concentration of 300 μM at 7 h before a challenging heat-treatment or irradiation in either cell line (Fig. 5), the induction of heat- and radio- resistance was strongly affected. After challenging exposures, heat resistance was very strongly suppressed and radio-resistance was almost completely suppressed.

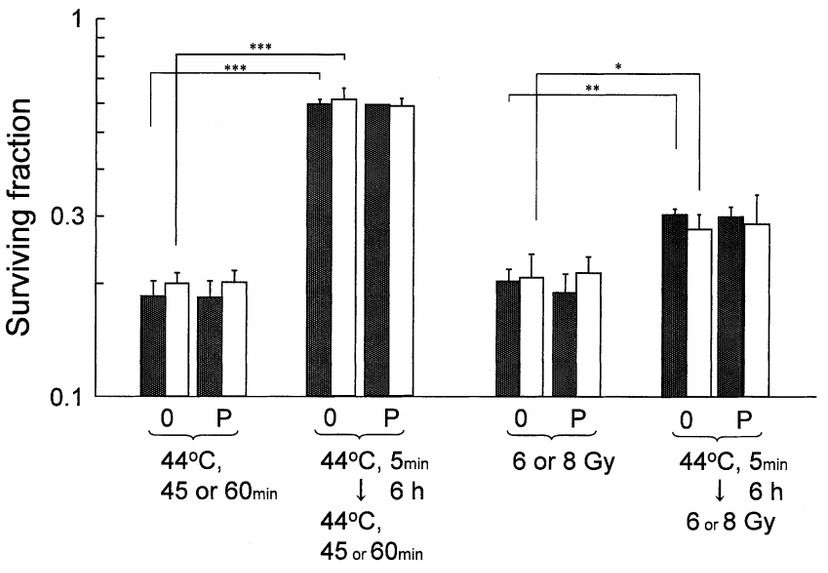

Fig. 4. Effect of a NO radical scavenger on the induction of heat- and radio-resistance. Cells were treated with heat shock or irradiation at 6 h after a priming heat treatment at 44°C for 5 min. H1299/wt*p53* cells (closed columns) and H1299/m*p53* cells (*o*pen columns) were heated at 44°C for 45 and 60 min, or irradiated with 6 and 8 Gy, respectively. Labels : **0** : no drugs added ; **P** : 10 μM c-PTIO (a NO radical scavenger). c-PTIO was added just before the challenging heat-treatment or irradiation. Asterisks (*, ** and ***) indicate statistically significant differences ($P<0.05$, 0.01 and 0.001, respectively) according to the Student's *t*-test.

Discussion

The induction of p53-independent heat- and radio-resistance by a priming heat treatment

The tumor suppressor gene *p53* plays a role as a guardian of genome integrity[12]. The activity of p53 is affected by exposure to many kinds of stress, and *p53* can determine the cell's fate in response to these stresses[22,23]. In fact, Fig. 1 shows that wt*p53* cells were more sensitive to heat or X-rays than m*p53* cells as previously reported[11,20]. In addition, Fig. 1*b* shows that wt*p53* cells were more radio-sensitive immediately after a priming heat treatment than were m*p53* cells as previously reported[19,20,24]. Moreover, previous work has demonstrated that the induction of heat- and radio-resistance resulted from a priming irradiation in wt*p53* cells, but not in m*p53* cells[9–11]. The work described here was intended to extend this work and to examine the existence of any *p53*-dependency on the induction of heat- and radio-resistance by a priming heat treatment. In the work reported here, radio- and heat-resistance were observed to be induced by a priming heat treatment in both cell lines, regardless of cellular *p53*-gene status (Fig. 1). The induction of heat- and radio-resistance also reached a maximum at 6 h after the priming heat treatment (Fig. 1). These results indicate that there is a definite interval in which a priming heat treatment can induce heat- and radio-resistance. There are two possible mechanisms which can be offered to explain the induction of heat- and radio-resistance by a priming heat treatment.

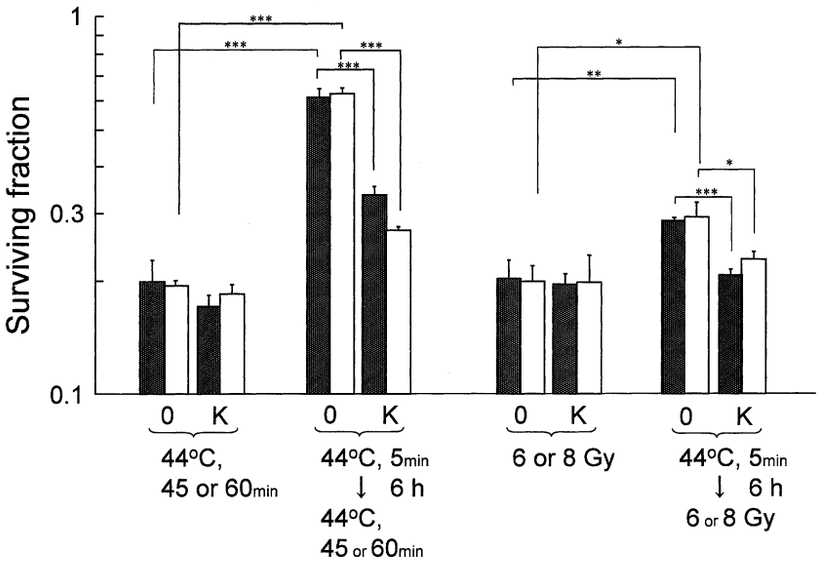

Fig. 5. Effect of a HSP inhibitor on the induction of heat- and radio-resistance. Cells were treated with heat shock or irradiation at 6 h after a priming heat treatment at 44°C for 5 min. H1299/wt*p53* cells (closed columns) and H1299/m*p53* cells (open columns) were heated at 44°C for 45 and 60 min or irradiated with 6 and 8 Gy, respectively. Labels : **0** : no drugs added ; **K** : 300 μM KNK437 (a HSP inhibitor). KNK437 was added to the culture medium at a final concentration of 0 or 300 μM 7 h before the start of a challenging heat-treatment or irradiation. The medium was exchanged for fresh medium 10 h after the start to challenging heat-treatment or irradiation. Asterisks (*, ** and ***) indicate statistically significant differences ($P<0.05$, 0.01 and 0.001, respectively) according to the Student's *t*-test.

HDM2, iNOS and NO radicals do not contribute to heat- and radio-resistance induced by a priming heat treatment

One reason to speculate that NO radicals might be involved in this process is the observation that the acquisition of heat- and radio-resistance in wt*p53* cells was observed after treatment with a NO radical donor at extremely low concentrations, in a manner similar to that seen for the induction of heat- and radio-resistance by a low dose priming irradiation[9–11]. Another work has shown that the treatment of cultured macrophages with several NO radical donors was able to reduce the micronuclei frequency induced by gamma irradiation. These observations suggested that NO radicals could act as a signal for repair system activation (*e.g.* for non-homologous recombination, and repair during S-phase) to reduce the micronuclei frequency[25]. In addition, soluble factors released from irradiated cells, such as NO radicals, appear to be important for the induction of radio-resistance[26]. It was recently shown that the induction of heat- and radio-resistance by a low dose priming irradiation was completely abolished by the addition of RITA (an inhibitor of p53-HDM2 interactions)[14], aminoguanidine (an iNOS inhibitor)[15] or c-PTIO (a NO radical scavenger)[16] to the culture medium of wt*p53* cells. Therefore, it was proposed that NO radicals are an initiator of heat- and radio-resistance following a pre-irradiation and acted

through the activation of HDM2, the depression of p53 accumulation, and the induction of iNOS.

The question of interest in this study was to examine the effect of RITA, aminoguanidine and c-PTIO on the induction of heat- and radio-resistance after a priming heat treatment. However, the induction of heat- and radio-resistance by a priming heat treatment was not suppressed by the addition of these compounds to the culture medium (Figs. 2, 3 and 4). Therefore, it is proposed that HDM2, iNOS and NO radicals do not contribute to the observed heat- and radio-resistance induced by a priming heat treatment.

HSPs contribute to the heat- and radio-resistance induced by a priming heat treatment

Another reason that HSPs can be thought to be involved in this process was the observation that the heat-resistance induced by a priming heat treatment (i.e. thermotolerance) is associated with the synthesis and cellular accumulation of a family of highly conserved proteins referred to as HSPs. It has been reported that Hsp27 and Hsp70 are the primary contributors to thermotolerance[3−5] through molecular chaperone activity[27,28]. It has also been recently shown that Polβ at least contributes to thermotolerance through its reactivation and stimulation by Hsp27 and Hsp70[21]. It has also been reported that the radio-resistance induced by a priming heat treatment, as well as thermotolerance, was associated with an increase in cellular Hsp70 levels[29]. To demonstrate the effect of HSPs on the induction of heat- and radio-resistance after a priming heat treatment, cells were exposed to KNK437, an inhibitor of HSPs[17] (Fig. 5). In the work reported here, the induction of heat- and radio-resistance by a priming heat treatment was suppressed by the addition of KNK437 to the culture medium (Fig. 5). These results indicate that HSPs likely contribute to induced heat- and radio-resistance observed after a priming heat treatment. Although it was reported that a low dose or a low dose-rate irradiation induced the expression of Hsp70[30−33], the induction of heat- and radio-resistance in cells was not observed in response to the inhibition of HSPs after a priming low dose irradiation (data not shown).

In summary, these observations provide support for the idea that a priming heat treatment induces HSPs, and a priming irradiation produced NO radicals which contribute to heat- and radio-resistance. These studies will hopefully contribute additional information and to the development of new models and to a further understanding of hyperthermic and radiation biology.

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