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Research paper

Nrf2 silencing to inhibit proteolytic defense induced by hyperthermia in HT22 cells

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ABSTRACT

Nrf2 pathway has been known to be protective against cancer progression however recent studies have revealed that the antioxidant activity of Nrf2 contributes to chemotherapy resistance. For many years, hyperthermia has been used as an additional therapy to increase the efficiency of chemotherapy and radiotherapy. Besides the positive effects of hyperthermia during treatment procedure, thermotolerance has been found to develop against heat treatment. Although the involved molecular mechanisms have not been fully clarified, heat shock proteins (HSP) and proteasome activity are known to be involved in the acquisition of thermotolerance. The aim of this study was to investigate the potential beneficial effects of combining hyperthermia with Nrf2 silencing to inhibit molecular mechanisms leading to induction of defense mechanisms in transcription level. Following heat treatment of HT22 cells, HSP70 and the proteasome levels and as well as proteasome activity were found to be elevated in the nucleus. Our results demonstrated that Nrf2 silencing reduced defense mechanisms against heat treatment both in antioxidant and proteolytic manner and Nrf2 may be a potential target for therapeutic approach in order to improve the beneficial effects of hyperthermia in cancer therapy.

1. Introduction

Hyperthermia (HT, thermal therapy, thermotherapy) which is used to destroy cancerous cells or to prevent their further growth, is a phenomenon that increases the body temperature with a range from 40 to 48 °C [1,2]. Hyperthermia is considered to be an effective cancer therapy approach particularly when combined with chemotherapy and radiotherapy [3]. During hyperthermia application, thermotolerance may develop against heat treatment via various pathways [4,5]. Thermotolerance is referred as the acquisition of a transient increased resistance to heat, which can be induced by a short exposure to a nonlethal heat treatment. Therefore, it is important to inhibit the molecular mechanisms that lead to thermotolerance in order to improve the antitumor efficiency of hyperthermia. Although these molecular mechanisms have not been fully clarified, induction of proteostasis markers such as heat shock proteins and proteasomal activity are known to be involved in thermotolerance [6].

Heat shock proteins (HSPs) exist in high concentrations in the cell and play a crucial role in sustaining protein homeostasis during cellular stress such as heat, oxidative stress, inflammation and irradiation [7]. They have four large and ubiquitous, ATP-dependent families classified as HSP100, HSP90, HSP70, and HSP60 proteins in addition to small HSPs [8]. HSP expression is tightly controlled in normal cells whereas it is enhanced by heat stress and is often dysregulated in tumor cells [9,10]. Hyperthermia is a protocol used to increase the efficiency of cancer therapy. The heat shock response following heat treatment may lead to the development of thermotolerance in tumor cells which reduces the beneficial effects of hyperthermia [6]. HSPs, especially HSP70 and HSP90, have been linked to this mentioned therapy resistance [11–13].

Proteasomal system is a crucial degradation pathway for the oxidatively modified proteins that makes it important for the

Abbreviations: ARE, Antioxidant Response Element; DNPH, 2,4-Dinitrophenylhydrazine; GAPDH, Glyceraldehyde 3-Phosphate Dehydrogenase; GCL, Glutamate Cysteine Ligase; GST, Glutathione S-Transferase; HDAC, Histone Deacetylase; HO-1, Heme Oxygenase 1; HSP, Heat Shock Protein; HT, Hyperthermia; MCA, Methylcoumarin; NQO1, NAD (P) H Quinone Oxidoreductase 1; Keap1, Kelch-like ECH-Associated Protein 1; Nrf2, NF-E2-Related Factor 2; PARP, Poly ADP Ribose Polymerase; TBST, Tris-Buffered Saline and Tween 20; UPS, Ubiquitin–Proteasome System

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removal of damage from the cell. It includes 20 S core particle and several regulators that influence the effect of degradation. Ubiquitin dependent system consists of 26 S multicatalytic protease which includes two 19 S regulatory particles and 20 S core particle in its structure [14]. Proteasome is responsible for the turnover of several proteins of intracellular origin [15,16]. It has been shown that the proteasomal system is responsible for the degradation of oxidized proteins [17–19]. Since most of the therapeutic approaches for cancer therapy induce oxidative damage of proteins, targeting proteasomal system is important to increase the efficiency of therapy. In addition, tumor cells have much higher proteasome activity, most of which seems to be localized in the nucleus when compared to nonmalignant cells. Thus, proteasomal degradation contributes to the development of resistance. Recently, the inhibition of the proteasome by specific inhibitors such as bortezomib has been a novel target for cancer chemotherapy since it has been shown to inhibit the growth of malignant tumor cells [20,21].

In cellular defense mechanism which is developed against oxidative damage, the transcription factor NF-E2-related factor 2 (Nrf2) controls transcriptional upregulation of antioxidant response element (ARE)-bearing genes, including those encoding endogenous antioxidants, phase II detoxifying enzymes and proteasome subunits [22]. While Nrf2 pathway has been known to be protective against cancer, recent work has revealed the “dark” side of Nrf2. However, the antioxidant activity of Nrf2 contributes to chemotherapy resistance by the induction of above mentioned genes that are involved in cell survival and growth [23].

To improve the efficiency of heat therapy, the inhibition of related defense mechanisms may be accepted as a novel strategy for cancer therapy. In our previous studies, we found that HSP70 and HSP90 protein expressions increase [8] in response to heat treatment in healthy young fibroblast cells related to the changes in proteasomal degradation. Our results lead us to hypothesize that the inhibition of hyperthermia-induced proteolytic defense mechanisms by Nrf2 silencing may be a useful approach since Nrf2 is known to regulate heat shock proteins and proteasome subunit expressions. In the present study, we tested the protein levels of HSP70, HSP40, HSP90, HO-1 and the proteasomal activity following the combination of hyperthermia and siRNA knockdown of Nrf2 in HT22 hippocampal tumor cell line. Our results revealed that targeting Nrf2 pathway may be a useful approach to overcome thermotolerance by the inhibition of defense mechanisms and may bring future aspects for clinical use of thermotherapy.

2. Material and methods

2.1. Cell culture and heat treatment

Mouse HT22 hippocampal cells were cultured in Dulbecco's modified Eagle's medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 10% fetal calf serum, and 0.35% glucose in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Cells initially were seeded at a density of 5×10^5 cells/ml and then grown for 3 days to a density of 3×10^6 cells/ml for protein expression analysis. 1×10^7 cells were grown to confluence for nuclear extraction analysis. Confluent cells were treated with mild hyperthermia at 42 °C for 1 h in culture medium and used immediately or used following culturing in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C for 1 h prior to analysis.

2.2. Lysis and immunoblotting of HT22 cells

To obtain whole cell lysates for immunoblotting analysis, cells were washed twice in 1 ml PBS and lysed with buffer containing 10 mM Tris HCl (pH 7.5), 0,9% NP-40, 0,1% SDS, 1 mM Pefabloc, and homogenized by passing through a 25-gauge needle ten times. Insoluble material was removed by centrifugation at 14,000 rpm for 20 min at 4 °C. The cytoplasmic and nuclear extracts were performed through the instructions of Nuclear Extract Kit (Active Motif, 40010). Protein concentrations were determined by BCA method.

Following the heat treatment and lysis of HT22 cells, 30 μg of whole cell lysates and nuclear lysates in reducing Laemmli-buffer (0.25 M Tris pH 6.8, 8% SDS, 40% glycerol, 0.03% bromophenol blue) were denatured at 95 °C for 5 min and applied to SDS-PAGE of 12% (w/v) acrylamide, followed by electrophoresis and blotted onto nitrocellulose membrane according to standard procedures. Immunodetections were performed with the following antibodies: anti-Nrf2 (D1Z9 C), anti-HSP90 (C45G5), anti-HSP70 (D69), anti-HSP40, anti-HO-1 (P249), anti-proteasome β5, anti-GSTα and anti-β-actin at 1:1000 dilutions. After exposure to peroxidase-coupled secondary antibodies (Bio-Rad) at 1:5000 dilution, membranes were developed using Lumi-Light western blotting substrate (Cell Signaling). Blots were visualized using X-ray film and quantified by densitometry using Image J software.

2.3. Detection of mRNA levels by quantitative RT-PCR

Total RNA isolation was carried out by RNA Mini Kit (Qiagen). The amount and purity of the RNA extracts were determined via Smartspec spectrophotometry (Bio-Rad). cDNA was synthesized by iScript cDNA Synthesis kit (Biorad) using 100 ng total RNA. Quantitative reverse transcriptase PCR was performed using Rotor Gene Q-RT PCR system (Qiagen), and QuantiTect PCR Sybr Green kit (Qiagen). PCR products were separated on 2.4% agarose gel in order to control the product base pair and the bands were extracted using QIAquick gel extraction kit (Qiagen). The dsDNA concentration of gel extractions were determined by spectrophotometry and 10^1–10^{10} dilutions prepared to study standard curve for quantitative analysis. The samples and the standard curve were obtained simultaneously in one run, and the results normalized to β-actin mRNA expression. The sequences of primers used were:

mouse Nrf2 forward, 5′-CTCGCTGGAAAAAGAAGTGG-3′;
mouse Nrf2 reverse, 5′-CCGTCCAGGAGTTCAGAGAG-3′;
mouse β-actin forward, 5′-AGCCATGTACGTAGCCATCC-3′;
mouse β-actin reverse, 5′-CTCTCAGCTGTGGTGGTGAA-3′.

2.4. Nrf2 siRNA transfection

HT22 cells were transiently transfected with siRNA targeting to Nrf2 by Accell siRNA delivery media (Dharmacon) according to the manufacturer's protocol. Briefly, 1.5×10^5 cells were plated into a 12 well plate and incubated at 37 °C with 5% CO_2 overnight. Regarding the number of the cells required for the nuclear extraction, all of the siRNA experiments were performed only in whole cells lysates and nuclear extraction was not performed with siRNA cells. 1 μM final concentration of Nrf2 siRNA (Smartpool, Dharmacon) in 1 × siRNA buffer diluted from 5 × siRNA buffer (Dharmacon) was added to the wells. The cells were incubated at 37 °C with 5% CO_2 for 72 h. The knockdown efficiency of Nrf2 was determined by QRT PCR analysis and 70% inhibition of Nrf2 mRNA level was detected. After 72 h of transfection, the cells were treated by hyperthermia at 42 °C for 1 h in delivery media and used immediately or following culturing in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C for 1 h prior to analysis. Then, the cells were lysed for immunoblotting analysis.

2.5. Proteasomal activity analysis by fluorimetry

The proteasome activity was measured in whole cell extracts, cytoplasmic and nuclear lysates of HT22 cells that are treated with hyperthermia. Peptidase activity toward the fluorogenic peptide substrate succinyl–leucine–leucine–valine–tyrosine–methylcoumaryl amide (suc-LLVY-MCA) was determined by incubation of samples with reaction buffer (150 mM Tris, 30 mM KCl, 5 mM MgOAc, 5 mM $MgCl_2$, 0.5 mM DTT, and 200 μM suc-LLVY-MCA) for 45 min at 37 °C. Fluorescence of the liberated methylcoumarin (MCA) was monitored using a micro plate reader at 360 nm excitation and 460 nm emission wavelengths. The amount of substrate degradation was calculated using the calibration curve prepared with free MCA standards as nmol MCA/mg proteinxmin.

2.6. Protein carbonyl measurement by Oxyblot

Following heat treatment, HT22 cells were lysed as explained above, 20 μg of total proteins were reduced by Laemmli-buffer and denatured at 95 °C for 5 min and applied to SDS-PAGE of 10% (w/v) acrylamide and followed by electrophoresis. The proteins for blotting were transferred onto a nitrocellulose membrane by Turbo Trans-blot system (Bio-Rad Laboratories). Following the electroblotting step, the membranes were equilibrated in TBS (100 mM Tris, 150 mM NaCl, pH 7.5) containing 20% methanol for 5 min, washed in 2N HCl for 5 min, incubated with 10 mM DNPH solution for 5 min, washed 3 × 5 min in 2N HCl and washed 5 × 5 min in 50% methanol. DNPH treated membrane was blocked with 5% non-fat dry milk in TBST (TBS contains Tween 20) for 1 h at room temperature with constant agitation. Blocked membrane was washed 3 × 5 min with TBST and incubated with rabbit anti-DNP antibody (Sigma) freshly diluted 1:2500 in 5% non-fat dry milk/TBST for 1 h at room temperature with constant agitation. Blotted membrane was washed 3 × 5 min with TBST and incubated with secondary anti-rabbit antibody (Bio-Rad) freshly diluted 1:5000 in 5% non-fat dry milk/TBST for 1 h at room temperature with constant agitation. The blotted membrane was washed 5 × 5 min with TBST. Membrane was developed using Lumi-Light western blotting substrate.

2.7. Statistical analyses

Statistical analysis was performed using Prism 4 (Graph-Pad) software. For determination of statistical significances of differences, one-way ANOVA was performed followed by multiple comparisons using the Student–Newman–Keuls test multiple comparison test. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Heat treatment increases nuclear protein expressions of Nrf2 in HT22 cells

The effects of hyperthermia at 42 °C for 1 h (without and with recovery at 37 °C for 1 h) on the protein expressions of Nrf2 in whole cell lysates and nuclear extracts were analyzed by immunoblotting (Fig. 1). Nrf2 protein expression was significantly increased by heat treatment that was reduced by recovery (Fig. 1A). This increase was more prominent in the nuclear protein expressions of Nrf2 following hyperthermia (Fig. 1B). To be able to confirm the purity of cytoplasmic and nuclear extracts, we performed immunoblotting of GAPDH and PARP (Fig. 1C). Interestingly, protein expressions of Nrf2 in the cytoplasmic extracts were

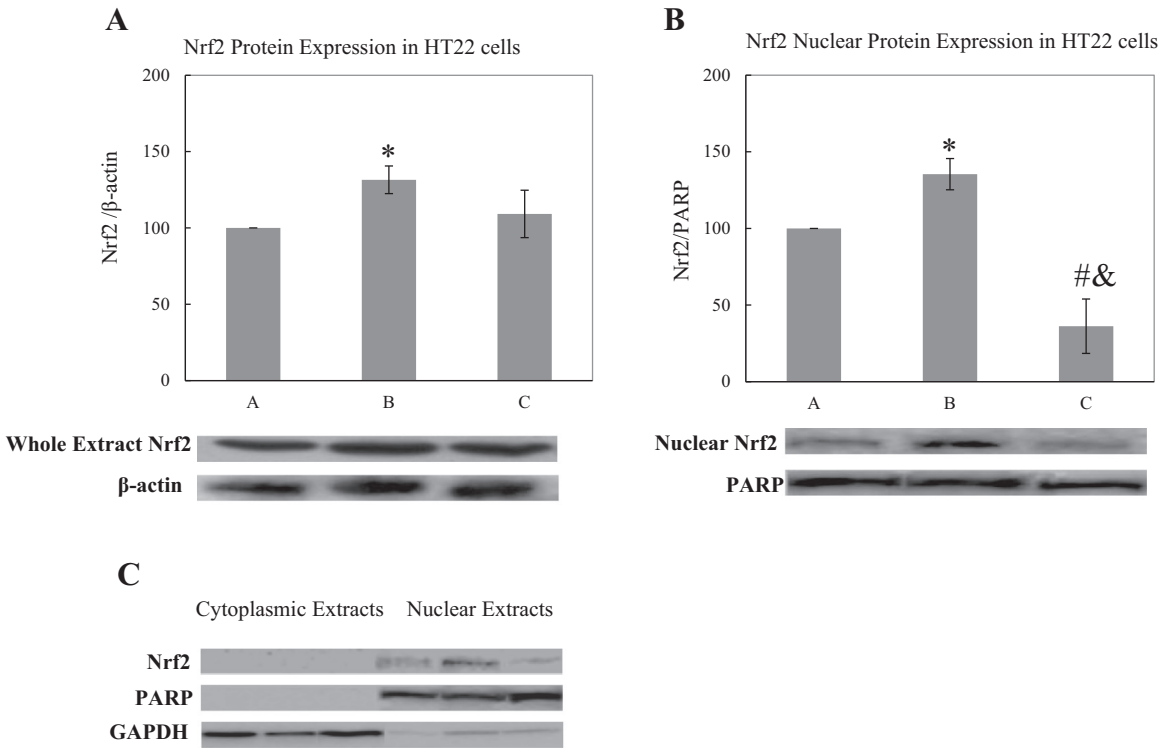

Fig. 1. Effects of heat stress on Nrf2 expressions in HT22 cells. Cells were treated with hyperthermia at 42 °C for 1 h and used immediately for analysis or cultured at 37 °C for 1 h as described in Materials and Methods. The groups of HT22 cells are represented as A: Control, B: 1 h of hyperthermia without recovery, C: 1 h of hyperthermia with recovery at 37 °C for 1 h for Fig. 1A and B. Data are represented as mean ± S.D., ($n=3$). (A) Representative immunoblots for Nrf2, β-actin expressions and densitometric analysis of Nrf2 protein expression in whole cell lysates relative to β-actin.*$P < 0.05$ for B *vs.* A. (B) Representative immunoblots for nuclear Nrf2, PARP expressions and densitometric analysis of Nrf2 nuclear protein expression relative to PARP. *$P < 0.05$ for B *vs.* A, #$P < 0.05$ for C *vs.* A, &$P < 0.05$ for C *vs.* B. (C) Representative immunoblots for Nrf2, GAPDH and PARP in cytoplasmic and nuclear extracts of HT22 cells.

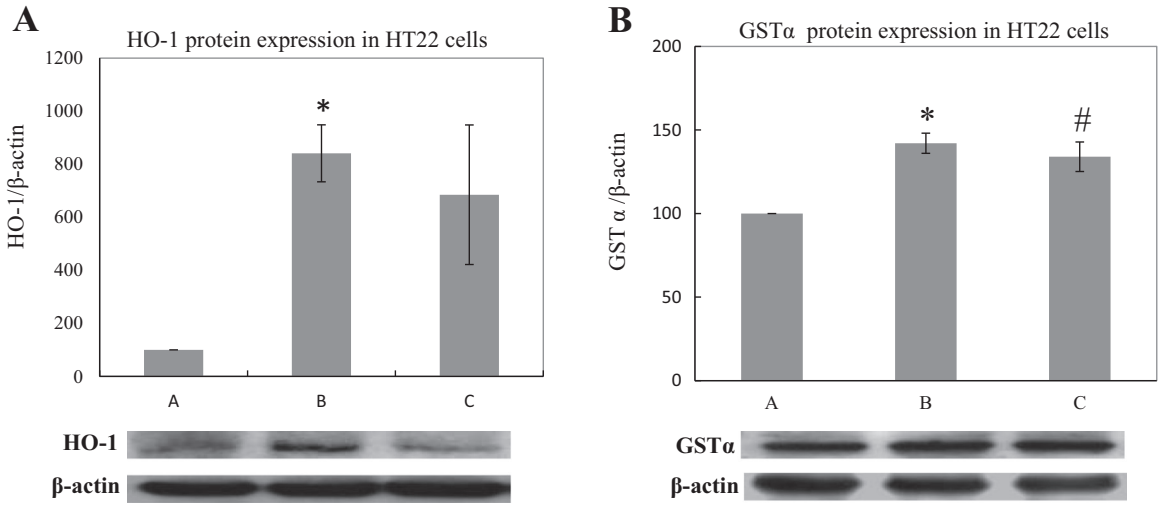

Fig. 2. Effects of hyperthermia and recovery on HO-1 and GSTα protein expressions. The groups of HT22 cells are represented as A: Control, B: 1 h of hyperthermia without recovery, C: 1 h of hyperthermia with recovery at 37 °C for 1 h for Fig. 2A and B. Data are represented as mean ± S.D., ($n=3$). (A) Representative immunoblots for HO-1 and β-actin expressions and densitometric analysis of HO-1 protein expression relative to β-actin in whole cell lysates. $^{*}P<0.05$ for B *vs.* A. (B) Representative immunoblots for GSTα and β-actin expressions and densitometric analysis of GSTα protein expression relative to β-actin in whole cell lysates. $^{*}P<0.05$ for B *vs.* A, $^{\#}P<0.05$ for C *vs.* A.

very low and Nrf2 is mostly located in the nucleus of HT22 tumor cells.

3.2. HO-1 and GSTα expressions increase following heat treatment

As target genes of Nrf2, HO-1 and GSTα protein expressions were analyzed following heat treatment. Both HO-1 and GSTα expressions were significantly induced by hyperthermia (Figs. 2A–B). These results demonstrated that the activation of antioxidant enzymes HO-1 and GSTα via Nrf2 pathway might play a role in cellular defense during heat treatment.

3.3. Heat treatment increases protein carbonylation in HT22 cells

Protein carbonyls are the most widely measured biomarkers for protein oxidation because of the relatively early formation and the stability of carbonylated proteins. The amount of protein bound carbonyls were measured following the heat treatment of HT22 cells according to Buss et al. [24]. The protein carbonyl content of cells was significantly enhanced with heat treatment that was reduced by 1 h recovery (Fig. 3A). Increase of the oxidative damage in the cell may be related to the upregulation of Nrf2 expression and proteasomal activity as defense mechanisms.

3.4. Hyperthermia induces both proteasome activity and the protein expression of proteasome subunit β5 in the nucleus

To test the role of hyperthermia in HT22 cells, proteasomal activity was measured by fluorometric detection method and we observed a significant increase in proteasomal activity of whole cells lysates which was decreased by 1 h recovery (Fig. 4A). However, the analysis of proteasomal activity in cytoplasmic and nuclear extracts showed that proteasome was activated in the nucleus and the activity was even decreased in the cytoplasm by hyperthermia (Fig. 4B–C). Consistent with our finding that Nrf2 expressions and proteasomal activity were increased in the nucleus of hyperthermia treated group, protein expression of proteasome subunit β5 was also significantly elevated in the nucleus when compared to control group (Fig. 4E).

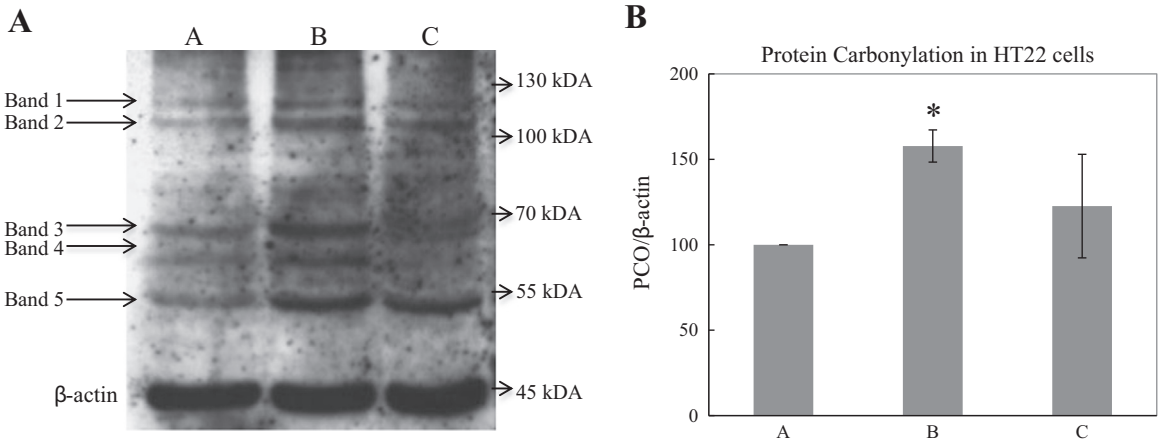

Fig. 3. Heat treatment increases protein carbonyl formation in HT22 cells. Cells were lysed and proteins were analyzed for the protein carbonyl formation measured with DNPH-reaction using a rabbit polyclonal anti-DNP (dinitrophenyl) primary antibody. The groups of HT22 cells are represented as A: Control, B: 1 h of hyperthermia without recovery, C: 1 h of hyperthermia with recovery at 37 °C. Data are represented as mean ± S.D., ($n=3$). (A) Representative immunoblot for Oxyblot and β-actin in whole cell lysates. (B) Densitometric analysis of protein carbonyl expression relative to β-actin. $^{*}P<0.05$ for B *vs.* A.

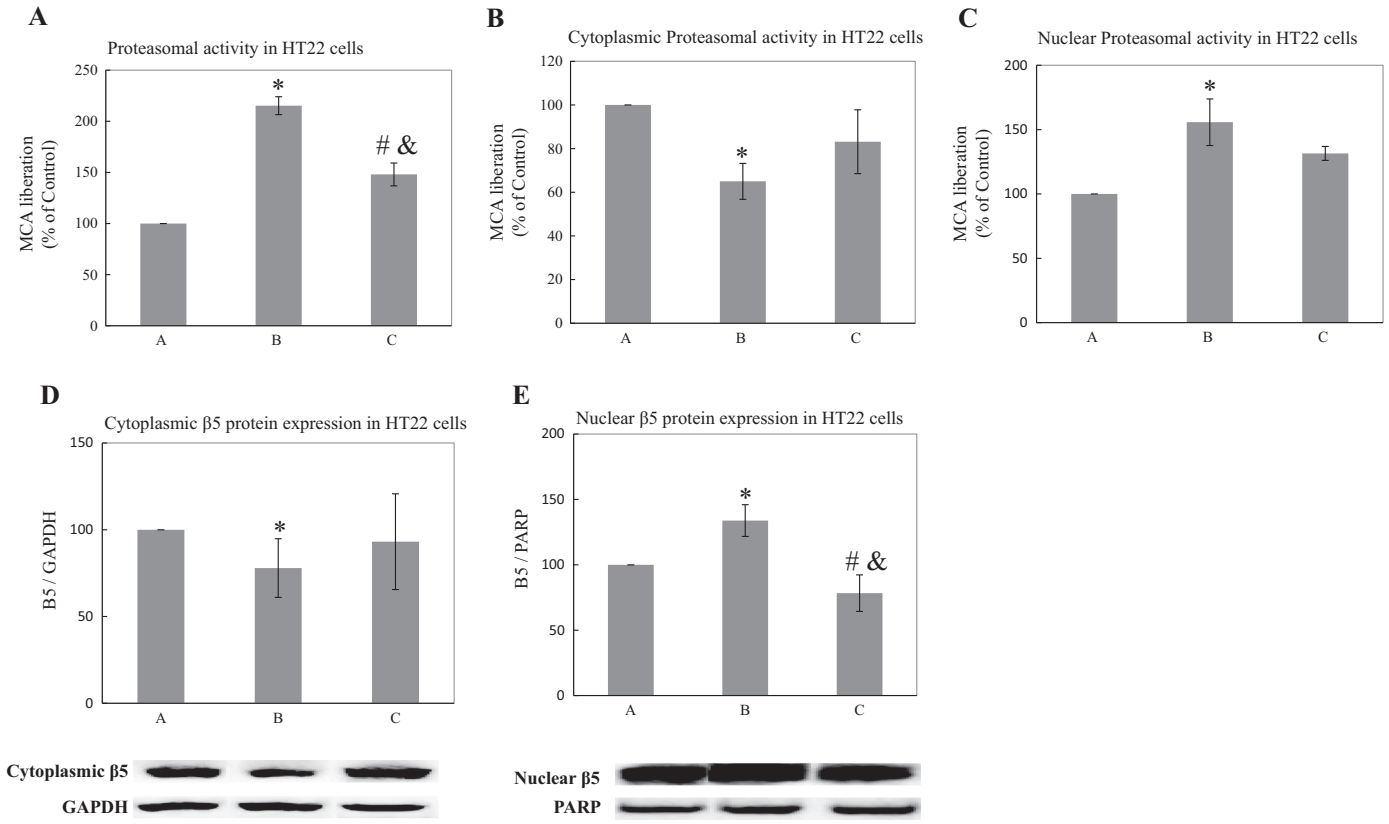

Fig. 4. Hyperthermia induces proteasome activity and β5 protein expression in the nucleus. The proteasome activity following heat treatment and recovery was measured in whole cell extracts, cytoplasmic and nuclear lysates of HT22 cells by fluorometric analysis (Fig. 4A, B and C). The activities in control lysates have been normalized to 100 nmol MCA/minxmg protein. Proteasome subunit β5 protein expression was determined by immunoblotting in cytoplasmic and nuclear lysates of HT22 cells (4D and 4E). The groups of HT22 cells are represented as A: Control, B: 1 h of hyperthermia without recovery, C: 1 h of hyperthermia with recovery at 37 °C for 1 h for Fig. 4A, B, C, D and E. Data are represented as mean ± S.D., (n=3). (A) The proteasomal activity in whole cell lysates. $^{*}P<0.05$ for B *v.s* A, $^{\#}P<0.05$ for C *vs.* A, $^{\&}P<0.05$ for C *vs.* B. (B) The proteasomal activity in cytoplasmic extracts $^{*}P<0.05$ for B *vs.* A. (C) The proteasomal activity in nuclear extracts.$^{*}P<0.05$ for B *vs.* A. (D) Representative immunoblots for proteasome subunit β5 and GAPDH expressions and densitometric analysis of proteasome subunit β5 protein expression relative to GAPDH in cytoplasmic extracts. $^{*}P<0.05$ for B *vs.* A. (E) Representative immunoblots for proteasome subunit β5 and PARP expressions and densitometric analysis of proteasome subunit β5 relative to PARP in nuclear extracts. $^{*}P<0.05$ for B *vs.* A, $^{\#}P<0.05$ for C *vs.* A, $^{\&}P<0.05$ for C *vs.* B.

3.5. The expressions of HSPs are affected by hyperthermia in HT22 cells

In order to gain information whether HSPs are affected by hyperthermia in HT22 cells, HSP70, HSP40 and HSP90 protein expressions were analyzed by immunoblotting (Fig. 5). HSP70 protein expressions were determined in cytoplasmic and nuclear extracts since HSP70 is known to translocate to the nucleus to interact with misfolded proteins [25]. Hyperthermia decreased the expression of HSP70 in cytoplasmic extracts (Fig. 5A) while it was significantly increased in nuclear extracts by both heat treatment and recovery when compared to controls (Fig. 5B). HSP40 expression was decreased by hyperthermia in correlation with reduced cytoplasmic HSP70 expression, was also significantly decreased in recovery group when compared to controls (Fig. 5C). These results are correlated with increased proteasomal activity that can be explained by the fact that HSP40 and HSP70 bind to the proteasome to target their substrates for the degradation. On the other hand, HSP90 protein expression was significantly increased by heat treatment in HT22 cells (Fig. 5D).

3.6. Nrf2 silencing decreases antioxidant defense by reduced HO-1 expression

In the present study, we showed that thermotholerance was gained against hyperthermia treatment either by antioxidant enzymes and proteolytic defense. Regarding our results, we aimed to inhibit thermotholerance by Nrf2 silencing. We treated cells with Nrf2 siRNA that are exposed to hyperthermia. Nrf2 silencing was checked by qRT-PCR analysis and we observed 70% reduction in Nrf2 mRNA expression when compared to control group (Fig. 6A). Consequently, 9-fold induction of HO-1 protein expression by hyperthermia (Fig. 2A) was reduced to 1.5 fold following Nrf2 siRNA transfection (Fig. 6B), confirming the role of Nrf2 in heat induced HO-1 expression.

3.7. Nrf2 silencing inhibits proteasome induction of heat treatment

When the cells were transfected with Nrf2 siRNA, the increase of proteasomal activity that is stimulated by heat treatment (Fig. 4A) was inhibited and there was no change between the groups (Fig. 7A). Moreover, in the same direction with the proteasomal activity results, Nrf2 silencing resulted in no significant change of proteasome subunit β5 expression between the groups (Fig. 7B) and induction of the nuclear β5 expression may be inhibited by Nrf2 siRNA transfection (Fig. 4E). In addition, we aimed to correlate the role of Nrf2 in HSP induced proteasomal degradation. In this direction, HSP40 and HSP90 protein expressions were determined in addition to HSP70 protein expression. Following Nrf2 siRNA treatment, there was no significant change of HSP70 protein expression between the groups (Fig. 8A). Following Nrf2 silencing, significant increase of HSP40 expression by heat treatment (Fig. 8B) may be resulted from its liberation from inhibited proteasome [26]. Nrf2

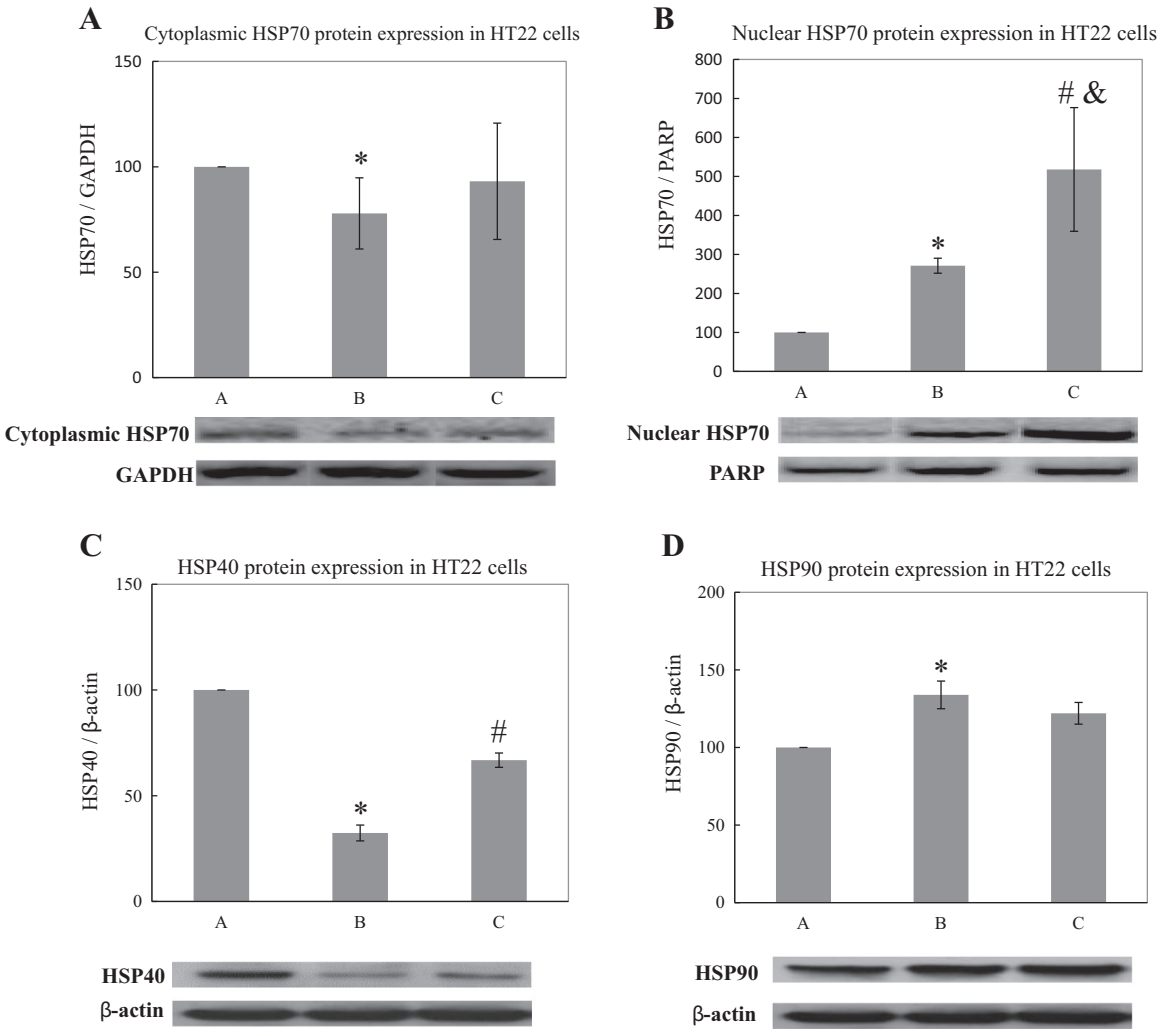

Fig. 5. Heat stress affects HSP expressions differently in HT22 cells. HSP70 protein expression was determined by immunoblotting in cytoplasmic and nuclear lysates. HSP40 and HSP90 protein expressions were determined by immunoblotting in whole cell extracts. The groups of HT22 cells are represented as A: Control, B: 1 h of hyperthermia without recovery, C: 1 h of hyperthermia with recovery at 37 °C for 1 h for Fig. 5A, B, C and D. Data are represented as mean ± S.D., ($n=3$). (A) Representative immunoblots for HSP70 and GAPDH expressions and densitometric analysis of HSP70 protein expression in cytoplasmic extracts relative to GAPDH.*$P<0.05$ for B *vs.* A. (B) Representative immunoblots for HSP70 and PARP expressions and densitometric analysis of HSP70 nuclear protein expression relative to PARP. *$P<0.05$ for B *vs.* A, #$P<0.05$ for C *vs.* A, &$P<0.05$ for C *vs.* B. (C) Representative immunoblots for HSP40 and β-actin expressions and densitometric analysis of HSP40 protein expression relative to β-actin in whole cell extracts.*$P<0.05$ for B *vs.* A, #$P<0.05$ for C *vs.* A. (D) Representative immunoblots for HSP90 and β-actin expressions and densitometric analysis of HSP90 protein expression in whole cell lysates relative to β-actin.*$P<0.05$ for B *vs.* A.

silencing was found to result in no significant effect on HSP90 protein expression (Fig. 6C).

4. Discussion

NF-E2-related factor 2 (Nrf2) signaling pathway is a main cellular detoxification program, has been regarded as beneficial for its antioxidant capacity for normal cells. However, recent work has undercut the notion that antioxidant activity is unambiguously protective against cancer progress [27]. In basal conditions, Nrf2 is inactivated in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1) which targets Nrf2 for ubiquitination and degradation by the proteasome [28]. Under stress conditions including oxidative, electrophilic or heat stress, Nrf2 is liberated from Keap1 which in turn translocates into the nucleus where it stimulates the expression of phase II and antioxidant enzymes including NAD(P)H quinone oxidoreductase 1 (NQO1), heme oxygenase 1 (HO-1), glutamanecysteine ligase (GCL) and glutathione S transferases (GSTs) [29–31]. Previously, our group showed that HSP70 protein levels were elevated in response to heat stress followed by recovery in young fibroblast cells [8]. This increase in HSP70 was related to, increased proteasomal activity. Thus, in the present study, we targeted Nrf2 silencing to evaluate possible contribution of Nrf2 in the development of proteasomal degradation response developed against hyperthermia in HT22 hippocampal tumor cell line.

Protein carbonyls are the most widely measured biomarkers of protein oxidation, as they are, in general, stable products formed relatively early during oxidative stress [18,32]. Protein carbonyl formation is also a good indicator of protein damage following cancer therapy [17,33]. Following heat treatment, protein carbonylation of the cells was measured by oxyblot as a biomarker of oxidative protein damage. We found a significant increase of protein carbonylation that was decreased after recovery of one hour in HT22 cells. Regarding the increase of oxidative stress, which is mediated by hyperthermia, we tested Nrf2 protein expression and its translocation to the nucleus. Nrf2 protein expression was found to be significantly elevated by heat treatment, which contributes, to the protection of cell via antioxidant

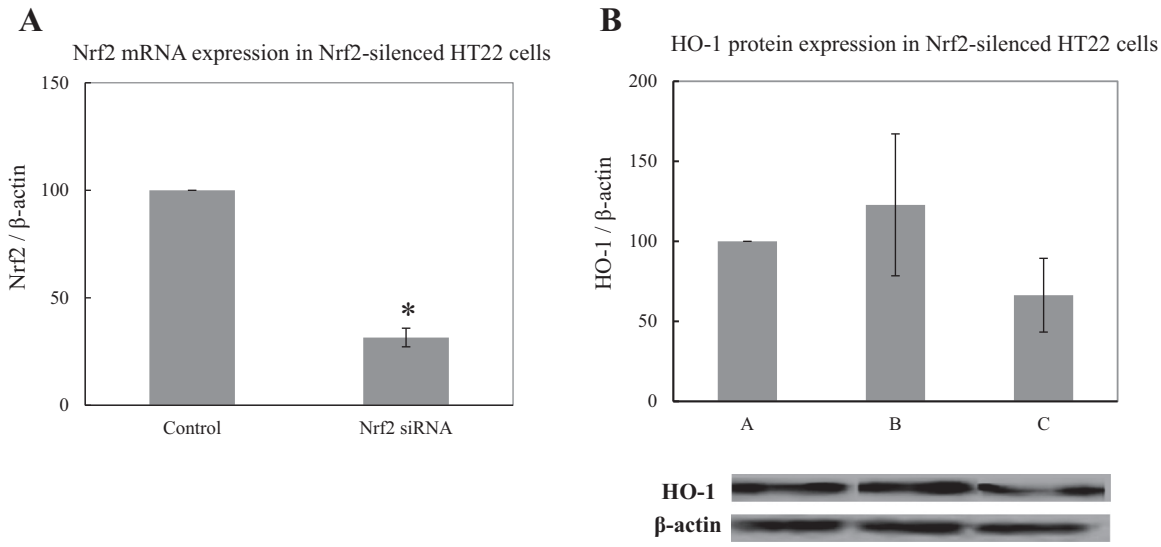

Fig. 6. Nrf2 siRNA decreased heat-induced HO-1 protein expression in HT22 cells. Data are represented as mean ± S.D., ($n=3$). (A) Densitometric analysis of Nrf2 mRNA expression relative to β-actin in Nrf2 siRNA transfected cells for the confirmation of the siRNA assay. The groups of HT22 cells are represented as A: Control, B: Nrf2 siRNA treated for Fig. 6A. *$P<0.05$ for Nrf2 siRNA *vs.* control. (B) Representative immunoblots for HO-1 and β-actin expressions and densitometric analysis of HO-1 protein expression relative to β-actin in Nrf2 siRNA transfected whole cell lysates. The groups of HT22 cells are represented as A: Nrf2 siRNA treated control, B: Nrf2 siRNA and 1 h of hyperthermia treated, C: Nrf2 siRNA and 1 h of hyperthermia with 1 h of recovery for Fig. 6B. No significant change between groups.

mechanisms. Interestingly, the increase of Nrf2 expression was originating from the nucleus and both in normal and heat stressed conditions, there was almost no Nrf2 protein in the cytoplasm. This showed that Nrf2 was already active in the nucleus of HT22 cells. The upregulation of antioxidant system in hyperthermia conditions was proved by elevated HO-1 and GSTα protein expressions, demonstrates that nuclear Nrf2 expression leads to the antioxidant cellular defense. Our data suggested the requirement of Nrf2 silencing and this may be a useful approach in aspect of suppressing the protection gained by antioxidant systems. In this direction, the decrease in the heat-induced HO-1 expression following siRNA transfection confirms the involvement of Nrf2 pathway via antioxidant mechanism in thermotolerance.

Moreover, Nrf2 may also be responsible for chemoresistance in terms of cellular proteostasis since Nrf2 regulates the expression of molecular chaperones [34], as well as of additional players of proteome stability and maintenance, including the proteasome subunits [35,36]. Although thermotherapy has proven to be an effective approach in cancer therapy, heat exposure induces synthesis and overexpression of heat shock proteins (HSPs) [37]. Therefore thermotolerance phenomenon depends on the ability of HSPs to function as molecular chaperones and prevent aggregation. Normally, heat stress can trigger programmed cell death via the induction of death signals but overexpression of HSPs in cancer cells following hyperthermia enhances tumor cell viability and impart increase resistance to subsequent chemotherapy [37]. The protection of cells against protein-damaging agents is provided by the increased HSPs both by affecting protein chaperoning and refolding and by directly blocking the cell-death pathways [38–40]. Additionally, the upregulation of HSPs frequently compromises the effects of thermotherapy [41,42]. Targeting HSP inhibition is an effective strategy to reverse induced defense

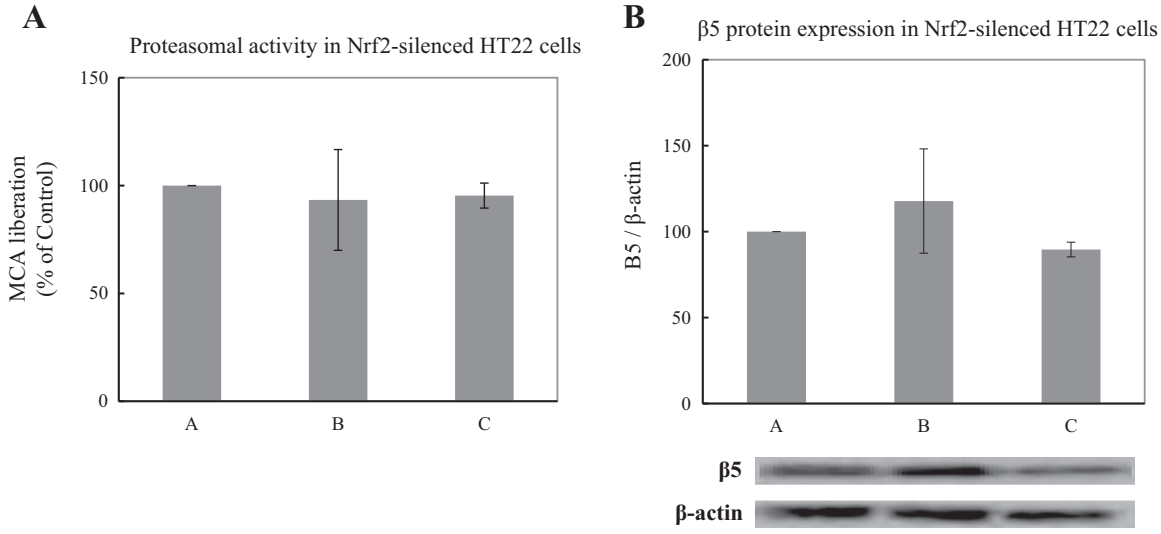

Fig. 7. Combination of Nrf2 silencing with hyperthermia inhibits thermotolerance gained by proteasome. The groups of HT22 cells are represented as A: Nrf2 siRNA treated control, B: Nrf2 siRNA and 1 h of hyperthermia treated, C: Nrf2 siRNA and 1 h of hyperthermia with1h of recovery for Fig. 7A and B. Data are represented as mean ± S.D., ($n=3$). (A) The proteasomal activity in HT22 cells transfected against Nrf2 siRNA. No significant change between groups. The activities in control lysates have been normalized to 100 nmol MCA/minxmg protein. (B) Representative immunoblots for proteasome subunit β5 and β-actin expressions and densitometric analysis of proteasome subunit β5 relative to β-actin in Nrf2 siRNA transfected whole cell lysates. No significant change between groups.

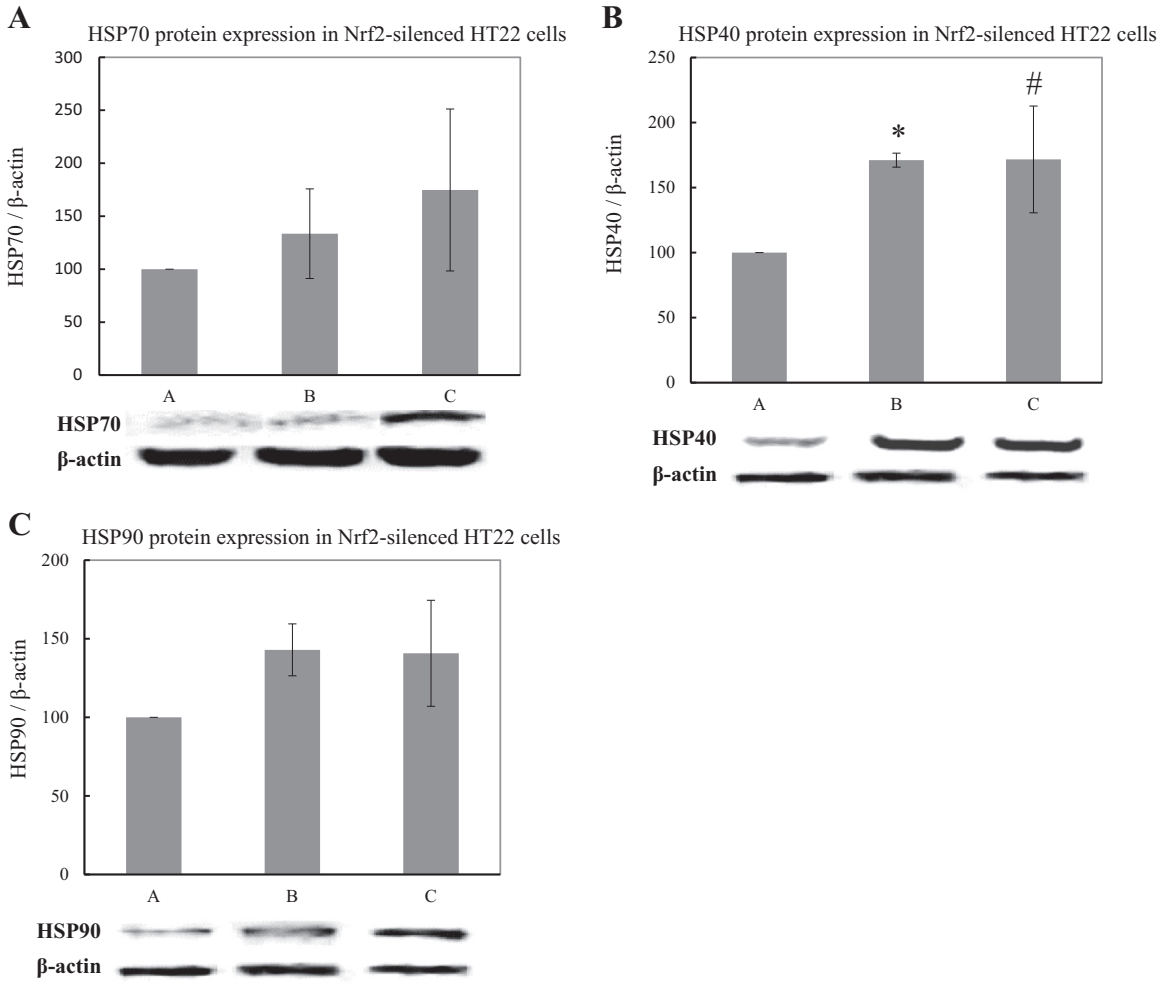

Fig. 8. The effects of Nrf2 silencing on the protein expressions of HSPs in heat-treated HT22 cells. The groups of HT22 cells are represented as A: Nrf2 siRNA treated control, B: Nrf2 siRNA and 1 h of hyperthermia treated, C: Nrf2 siRNA and 1 h of hyperthermia with 1 h of recovery treated for Fig. 8A, B and C. Data are represented as mean ± S.D., ($n=3$). (A) Representative immunoblots for HSP70 and β-actin expressions and densitometric analysis of HSP70 protein expression relative to β-actin in Nrf2 siRNA transfected whole cell lysates. No significant change between groups. (B) Representative immunoblots for HSP40 and β-actin expressions and densitometric analysis of HSP40 protein expression relative to β-actin in Nrf2 siRNA transfected whole cell lysates. $^{*}P < 0.05$ for B *vs.* A, $^{\#}P < 0.05$ for C *vs.* A. (C) Representative immunoblots for HSP90 and β-actin expressions and densitometric analysis of HSP90 protein expression relative to β-actin in Nrf2 siRNA transfected whole cell lysates. No significant change between groups.

mechanisms of cancer cells [41,42] and we aimed to inhibit heat related proteasome activation directly from the transcriptional process via Nrf2 pathway. In our study, we observed increased HSP90 expression following heat treatment. Interestingly, HSP70 protein expression was increased in nuclear extracts by heat treatment showed that in correlation with Nrf2, HSP70 was also active in the nucleus. The decrease in cytoplasmic HSP70 expression may be due to the translocation of HSP70 to the nucleus and HSP70 translocation was also confirmed in other studies for the protection of DNA against several conditions [25,43]. Together with the decrease of HSP70 in the cytoplasm, HSP40 levels were also decreased in the cells. These results are consistent with previous studies, which showed that HSP expressions may be induced or repressed in different cell lines [44] and repression is generally accompanied by the translocation into the nucleus [45]. In the present study, thermotolerance gained by elevated HSP70 expression and related proteasome activation was inhibited following Nrf2 siRNA treatment. Nrf2 silencing resulted in a decrease of hyperthermia-induced HSP70 expression and there was no significant change between the groups. Meanwhile, Nrf2 silencing did not affect HSP90 expression significantly. Decreased HSP40 expression by hyperthermia was found to be induced following Nrf2 siRNA treatment, which may be explained by the inhibition of proteasomal degradation.

In mammalian cells, a range of proteases exists which are distributed throughout the cell and the major proteolytic system responsible for the removal of oxidized proteins is the proteasomal system [46]. 26 S proteasome is a multicomponent proteinase responsible for the turnover of the majority of proteins of intracellular origin [15,16] and it can control the expression of many relatively short-lived regulatory proteins involved in cell growth and proliferation. Originally thought of as an indiscriminate protein digestive system, it is now recognized as a highly coordinated regulatory pathway that is present in the nucleus and in the cytoplasm [47]. Recently, the discovery that specific inhibitors of the ubiquitin proteasome pathway inhibit the growth of malignant tumor cells has led to a growing interest in the proteasome as a novel target for cancer chemotherapy [20]. On the other hand, proteasome inhibition was shown to induce the elevation of Nrf2 and HO-1 in a HDAC6 coordinated mechanism that could impair the efficiency of proteasome inhibitor treatment [48–50]. In the current study, we both analyzed the proteasomal activity and

proteasome β5 expression following heat treatment and Nrf2 siRNA transfection. Proteasomal activity was significantly increased in the whole cell lysates by hyperthermia and was decreased by 1 h recovery. However, the analysis of proteasomal activity in cytoplasmic and nuclear extracts showed that proteasome was activated in the nucleus and the activity was decreased in the cytoplasm by hyperthermia. This may be explained together with the translocation of HSP70 to the nucleus and also the role of HSP70 for the association of 26 S proteasome for the activation [26]. When the cells were transfected with Nrf2 siRNA, the increase of proteasomal activity that was stimulated by heat treatment was inhibited. Similarly, nuclear expression of proteasome subunit β5 was also elevated by heat treatment and Nrf2 silencing reduced the significant increase of β5 expression. Depending on our results, in the same direction with Nrf2 expression, both proteasomal activity and proteasome expression were found to be more active in the nucleus and it seems proteasome translocates to the nucleus in heat stress conditions. Consequently, their activation by heat stress was inhibited in whole cell lysates by Nrf2 silencing. These results supported our previous data that oxidative damage in HT22 tumor cells that was supposed to be also a consequence of chemotherapy was ameliorated by nuclear proteasome [17] which was inhibited by Nrf2 pathway in our study.

The results from various studies have shown that hyperthermia is both ideal complementary treatment to, and a strong sensitizer of, both chemo- and radiotherapy [51,52]. Additionally, the expectations raised by the experimental studies have been confirmed by the results from clinical studies [53–55]. In spite of the remarkable therapeutic gain that has now been demonstrated in patients, hyperthermia is still not widely recognized as a useful treatment due to the development of thermotolerance. In this point, Nrf2 may contribute to improve the effectiveness of hyperthermia by the inhibition of defense mechanisms that are induced during the therapy in several ways. In the present study, Nrf2 silencing inhibited both proteasome activity and expression which contributes to acquisition of thermotolerance. Regarding our results, an inhibitor of Nrf2 can be used in conjunction with thermotherapy to sensitize cancer cells to chemical treatment. Since Nrf2 is proved to regulate the expression of several downstream genes related to antioxidant systems and cellular proteostasis protecting cancer cells, it would be a more efficient drug target than the individual downstream gene.

5. Conclusion

In conclusion, we showed that targeting Nrf2 might be a useful approach for the acquisition of thermotolerance through inhibition of various defense mechanisms. Our results demonstrated that these mechanisms that are affected by Nrf2 activation are the antioxidant players such as HO-1 and GSTα and players of protein turnover including HSPs and the proteasomal system. In addition, we showed that all of these factors are mostly active in the nucleus of the cell. Therefore, in order to inhibit development of thermotolerance gained by proteasome and antioxidant systems, repression of Nrf2 signaling pathway would contribute to the efficiency of hyperthermic therapy in human cancers.

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