Biochimica et Biophysica Acta 1853 (2015) 52–62

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Thermotolerance induced at a mild temperature of 40 °C alleviates heat shock-induced ER stress and apoptosis in HeLa cells

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ARTICLE INFO

Article history:
Received 14 August 2014
Received in revised form 16 September 2014
Accepted 17 September 2014
Available online 26 September 2014

Keywords:
Hyperthermia
ER stress
Apoptosis
Calpain
Caspase
Hsp72

ABSTRACT

Hyperthermia (39–45 °C) has emerged as an alternate prospect for cancer therapy in combination with radiation and chemotherapy. Despite promising progress in the clinic, molecular mechanisms involved in hyperthermia-induced cell death are not clear. Hyperthermia causes protein denaturation/aggregation, which results in cell death by apoptosis and/or necrosis. Hyperthermia also induces thermotolerance, which renders cells resistant to subsequent exposure to lethal heat shock. This study investigates the role of both lethal (42–43 °C) and mild (40 °C) hyperthermia in regulating ER stress and ER stress-induced apoptosis in HeLa cells. The ability of mild thermotolerance induced at 40 °C to alleviate either or both of these processes is also determined. Hyperthermia (42–43 °C) induced ER stress, revealed by phosphorylation of PERK, eIF2α and IRE1α, cleavage of ATF6 and increased expression of BiP and sXBP1. Real-time PCR revealed that mRNA levels of ATF6, ATF4, BiP, sXBP1 and CHOP increased in cells exposed to hyperthermia. Moreover, hyperthermia caused disruption of calcium homeostasis and activated the calpain-calpastatin proteolytic system and ER resident caspase 4. Pre-exposure to mild hyperthermia (40 °C) alleviated the induction of cytotoxicity and ER stress by hyperthermia (42–43 °C) and protected cells against ER stress-induced apoptosis. ShRNA-mediated depletion of Hsp72 abrogated protective effects of mild thermotolerance (40 °C) against heat-shock induced ER stress and sensitized cells to ER stress-mediated apoptosis. Our findings show that Hsp72 contributes to the protective effects of mild hyperthermia (40 °C) against hyperthermia-induced ER stress and apoptosis.

1. Introduction

Over the past decades, the struggle against cancer has led to the discovery of new strategies to fight this disease and to bring hope to patients. Among these new strategies, hyperthermia (39–45 °C) (also known as thermal therapy or thermotherapy) has emerged as a promising alternative that can treat a wide range of tumor types with minimal injury to normal tissues [1]. Furthermore, unlike healthy cells, tumors act as a heat reservoir when subjected to local hyperthermia. Tumors are unable to increase blood flow in response to thermal stress, which makes them more vulnerable to heat damage than the surrounding normal tissue [1,2]. This results in collapse of the tumor vascular system and destruction of tumor cells. In addition, hyperthermia appears to be a potent modifier of tumor response to radiation and several chemotherapy agents by increasing and targeting their cytotoxic effects in the tumor volume. A multitude of randomized studies showed that hyperthermia combined with radiotherapy, chemotherapy or both, resulted in significant improvement in clinical outcome in cancer patients [1–7]. The tumor sites include cervix, soft-tissue sarcoma, breast, head and neck, rectum, brain, bladder, lung, esophagus, liver, appendix, prostate and melanoma. Several larger phase III randomized trials showed that hyperthermia improved the rate of clinical complete response in patients treated with radiotherapy for superficial breast cancer and chest wall recurrence [7]. Hyperthermia was beneficial when combined with re-irradiation for breast cancer recurrences [5]. For high-risk soft tissue sarcoma, the addition of regional hyperthermia to a multimodal treatment of surgery, radiotherapy, and chemotherapy was shown to improve local recurrence- and disease-free survival [4]. Several randomized trials showed an improvement by adding

Abbreviations: ASK1, apoptotic-signaling kinase-1; ATF4, activating transcription factor 4; ATF6, activating transcription factor 6; Bcl-2, B cell lymphoma 2; BiP, binding immunoglobulin protein; cATF6, cleaved ATF6; CHOP, transcriptional factor C/EBP homologous protein; DMEM, Dulbecco's modification of Eagle's medium; DTT, dithiothreitol; eIF2α, eukaryotic translation initiation factor; ER, endoplasmic reticulum; GADD34, growth arrest and DNA damage-inducible 34; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HeLa, human cervical carcinoma cells; HRP, horseradish peroxidase; Hsp, heat shock protein; IRE1α, inositol-requiring protein-1; JNK, c-Jun N terminal kinase; MOPS, 3-(N-morpholino)-propane sulfonic acid; PBS, phosphate-buffered saline; PERK, protein kinase RNA (PKR)-like ER kinase; PI, propidium iodide; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; SEM, standard error of mean; sXBP1, spliced XBP1; TBP, TATA-box binding protein; TT, thermotolerant; XBP1, X-Box binding protein 1

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http://dx.doi.org/10.1016/j.bbamcr.2014.09.016
0167-4889/

hyperthermia to radiation for cervical cancer patients, even at 12 years follow-up [3,6].

Despite promising progress in the clinic, the mechanisms involved in heat stress-induced cell death are not well understood [8]. Hyperthermia causes many changes in cells as well as a loss of cellular homeostasis [9–13]. A key event appears to be protein denaturation and aggregation [14], which results in cell cycle arrest, inactivation of protein synthesis, and inhibition of DNA repair processes. The correct structure and conformation of proteins is essential for their function in the cell. A small increase in temperature can cause protein unfolding, entanglement and aggregation leading to an imbalance in proteostasis. This can result in increased degradation of aggregated/misfolded proteins through the proteasomal and lysosomal pathways. Other cellular effects of hyperthermia include the: (1) inhibition of DNA synthesis, transcription, RNA processing and translation; (2) disruption of the membrane cytoskeleton; (3) metabolic changes (e.g. uncoupling of oxidative phosphorylation) that lead to decreased ATP levels; and (4) alterations in membrane permeability that cause increases in intracellular levels of Na^+, H^+ and Ca^{2+} [9,10,12]. Furthermore, changes occur to intracellular organelles: the Golgi system and endoplasmic reticulum (ER) were fragmented during heat shock and modest swelling of mitochondria occurred [15].

The ER is an organelle that is highly responsive to the nutrient and energy status of the cell and plays an important role in the folding of newly synthesized proteins. When the folding capacity of the ER is exceeded, misfolded/unfolded proteins accumulate and lead to ER stress [16]. Cells use an adaptive mechanism to counter the deleterious effects of ER stress, known as the unfolded protein response (UPR) [17]. The UPR consists of three major branches that are controlled by the ER transmembrane proteins PKR-like ER-regulated kinase (PERK), inositol requiring protein 1α (IRE1α) and activating transcription factor 6 (ATF6) [18–20]. In particular, the PERK/eukaryotic translation initiation factor 2α (eIF2α) sub-arm of ER stress signaling is critical for the UPR in cancer cells and their adaption to hypoxia, as well as their resistance to therapy [21]. PERK phosphorylates eIF2α, which inhibits general protein translation. This allows selective translation of ATF4, which activates the transcription of ER chaperones such as BiP. The distant UPR arms (IRE1α/X-Box binding protein 1 (XBP1)) synergize to attenuate stress by increasing the folding capacity of the ER [16]. IRE1α catalyzes the alternative splicing of *XBP1* mRNA, leading to expression of the sXBP1 transcription factor, which activates ER chaperone genes. The third arm is mediated through the transcription factor ATF6. ATF6 undergoes proteolysis in the Golgi apparatus which leads to activation followed by its translocation to the nucleus. One of the ATF6 target genes is *XBP1*. However, if the compensatory mechanisms fail to facilitate the adaptation of cells to ER stress, induction of the UPR can lead to the elimination of stressed cells by apoptosis [22–24]. C/EBP homologous protein (CHOP), also known as growth arrest- and DNA damage-inducible gene 153 (GADD153), plays a convergent role in the UPR and is as an important mediator of ER stress-induced apoptosis [24,25]. CHOP-mediated activation of growth arrest and DNA damage-inducible 34 (GADD34) promotes protein dephosphorylation of eIF2α, reversing the translational inhibition to allow the recovery of protein synthesis following a stress insult.

The induction of apoptosis following ER stress can occur in various ways including the activation of caspase 12/4, cleavage of transmembrane ER protein Bcl-2-associated protein-31 (BAP31) by active caspase 8, and the activation of c-Jun N terminal kinase (JNK) via the apoptosis signaling kinase 1 (Ask1)/IRE1α/TNF receptor-associated factor 2 (TRAF2) complex [24,26]. In addition, cytosolic free calcium levels have been reported to increase during ER stress-induced apoptosis, thereby leading to the activation of calpains and subsequent cleavage of the anti-apoptotic B cell lymphoma 2 (Bcl-2) family member Bcl-X_L [27]. Besides these direct pathways, activation of caspase 8 and/or caspase 7 may occur during ER stress-induced apoptosis, leading to cytochrome c release and caspase 9 activation. Additionally, ER stress may activate the traditional mitochondrial pathway through a crosstalk between both compartments involving the Bcl-2 family proteins [28].

Hyperthermia is cytotoxic at temperatures above 42.5 °C and selectively lethal to cancer cells while mild hyperthermia is known to be beneficial to tissues and organs [1]. Hyperthermia-range temperatures cause protein denaturation and aggregation [9,14] and therefore, it is likely that hyperthermia could induce ER stress. We previously reported that heat preconditioning at a mild temperature (40 °C for 3 h) in human adenocarcinoma cervical HeLa cells led to the development of mild thermotolerance, which was associated with an increase in the expression of several heat shock proteins (Hsp) including Hsp72 [29]. This work aims to determine whether mild doses of hyperthermia (40 °C) can activate the ER stress survival response, and whether more severe heat exposure can tip the balance towards ER stress-mediated apoptosis. This study also determines whether pre-conditioning with mild hyperthermia (40 °C) could protect cells against ER stress-mediated apoptosis induced by lethal hyperthermia (42–43 °C), and whether Hsp72 plays a role in this adaptive survival response.

2. Experimental procedures

2.1. Reagents

Dulbecco's Modified Eagle Medium (DMEM), penicillin/streptomycin, fetal bovine serum (FBS), trypsin and Fluo 3-AM (1-[2-Amino-5-(2,7-dichloro-6-hydroxy-3-oxo-9-xanthenyl)phenoxy]-2-(2-amino-5-methylphenoxy)) were purchased from Invitrogen (Carlsbad, CA). Antibodies for tubulin were purchased from Upstate Biotechnology (Lake Placid, NY). Antibodies for pPERK (Thr980), PERK, peIF2α (Ser51), eIF2α, sXBP1, ATF6, IRE1α, BiP, caspase 4 and vinculin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) while calpain, calpastatin and cleaved caspase-3 antibodies were from Cell Signaling (Beverly, MA). Antibodies for pIRE1α (Ser724) were purchased from Abcam (Cambridge, MA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from BioResources International (Carlsbad, CA). BAPTA-AM (1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester), Hoechst 33258, propidium iodide (PI), sulforhodamine B and all other chemicals, unless otherwise indicated, were purchased from the Sigma Chemical Co. (St. Louis, MO).

2.2. Cell culture

HeLa cells (ATCC #CCL-2) were grown in monolayer in DMEM containing 10% FBS, 50 U/ml penicillin and 50 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 in a water jacketed incubator [30]. The cells were grown to near confluence and cell culture medium was replaced with fresh medium 24 h before experiments. To induce thermotolerance (TT), cells were grown to near confluence, culture medium was replaced with fresh medium for 24 h, and then confluent cells were transferred to an identical incubator for 3 h at 40 °C (±0.1 °C), following a period of 20 min to allow the temperature of the culture medium to reach 40 °C [26]. Confluent cells were harvested using 0.25% (w/v) trypsin-0.02% (w/v) EDTA solution, and washed by centrifugation (1000×*g*, 3 min).

At 40 °C, maximum levels of thermotolerance and induction of Hsps occur after 3 hours [29]. Protein synthesis continues at 40 °C and does not stop for several hours as occurs when thermotolerance is induced by exposure to higher temperatures such as 43–45 °C. There was no loss of viability in cells heated at 40 °C for 3 h, evaluated by trypan blue exclusion (data not shown).

Hsp72 silencing in HeLa cells was achieved by testing four different hairpins. Packaging (psPAX2) and envelope (pMD2.G) vectors were obtained from Addgene (Boston, MA). Lentiviruses were generated by co-transfection of vectors in 293FT cells (#R700-07, Life Technologies) using Lipofectamine 2000 (Invitrogen) following manufacturer's

guidelines and then used to infect HeLa cells. Cells were selected using puromycin (2 μg/ml) and drug-resistant pools were propagated.

2.3. Heat treatment

Freshly harvested thermotolerant (3 h at 40 °C) and non-thermotolerant cells (3 h at 37 °C) in phosphate-buffered saline (PBS)-1% bovine serum albumin (BSA) supplemented with 10 mM of glucose in a final volume of 1.0 ml were heated for 3 h at 42 or 43 °C, relative to controls at 37 °C, in temperature-controlled precision waterbaths (±0.02 °C) (Haake D8, Fisher Scientific, Montreal, QC) [29]. One ml of cell suspension reached a temperature within 0.1 °C of the waterbath temperature within 3 min. There was no recovery period between development of thermotolerance (3 h at 40 °C) and hyperthermia treatment at 42–43 °C.

2.4. Cytotoxicity

Cytotoxicity assays were performed using sulforhodamine B to stain cellular proteins as described previously [29]. Freshly harvested normal and thermotolerant cells (5×10^6/ml) were incubated in 1 ml of PBS-1% BSA supplemented with 10 mM glucose in tubes in a waterbath (Haake D8) at 43 °C for up to 180 min relative to controls at 37 °C. The cells were then diluted and seeded in 96-well microplates in DMEM medium and incubated at 37 °C for 96 h. The cells were fixed with 17% trichloroacetic acid in PBS and cellular protein was stained with 0.4% sulforhodamine B in 1% acetic acid. Quantification of sulforhodamine B was carried out by spectrofluorimetry using a UV max kinetic microplate reader (Spectra Max Gemini, Molecular Devices, Sunnyvale, CA) at a wavelength of 540 nm. The relative plating efficiency of each cell type was determined by dividing the absorbance observed for a given treatment by the absorbance detected in the normal cells incubated at 37 °C for 180 min and expressed as a percentage.

2.5. Morphological assessment of apoptosis

Briefly, thermotolerant and non thermotolerant cells were heated at 42 and 43 °C for 3 h. Where indicated, cells were pretreated for 2 h with 100 μM calcium chelator BAPTA-AM (Sigma-Aldrich), 20 μM calpain inhibitor I (Ac-LLnL-CHO) (Sigma-Aldrich), 20 μM caspase-4 inhibitor (LEVD-CHO) or 50 μM caspase-7/3 inhibitor I (5-[(S)-(+)-2-(methoxymethyl)pyrrolidino]sulfonylisatin) (Calbiochem, La Jolla, CA). Thereafter, Hoechst 33258 and propidium iodide (PI) were added to visualize apoptotic and necrotic cells, respectively, by fluorescence microscopy [29,30]. For each image, at least 300 cells were counted using Northern Eclipse software (Empix Imaging Inc., Mississauga, ON). The percentages of apoptotic and necrotic cells were determined relative to total cells (obtained using bright field illumination).

2.6. Biochemical analyses

Cells were homogenised in radio-immunoprecipitation assay buffer (RIPA: 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 1% sodium deoxycholate, 5 mM EDTA, 1 mM NaF, 1 mM sodium orthovanadate and protease inhibitors). Lysates were clarified by centrifugation at 13,000 rpm for 10 min, and protein concentrations were determined using a bicinchoninic acid assay kit (Pierce Chemical Co.). Proteins (30–90 μg) were resolved by SDS-PAGE and transferred to PVDF membranes [29,30]. Immunoblotting of lysates was performed with primary antibodies, and after incubation with secondary antibodies, proteins were visualized using enhanced chemiluminescence (Amersham Biosciences Corp., Piscataway, NJ). Pixel intensities of immunoreactive bands were quantified using FluorChem Q Imaging software (Alpha Innotech).

Total RNA was extracted from cells using TRIzol reagent (Invitrogen). mRNA of PERK, IRE1α, ATF6, GADD34, BiP, spliced (s) XBP1, CHOP, and ATF4 was assessed by quantitative reverse transcription PCR (iCycler, Bio-Rad Laboratories, Hercules, CA) with appropriate primers (Integrated DNA Technologies, Coralville, Iowa) (Table 1) and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

For nuclear preparations, cells were washed in buffer A [100 mM sucrose, 1 mM EGTA, 20 mM 3-(N-morpholino)-propane sulfonic acid (MOPS), pH 7.4] and then resuspended in buffer B [buffer A plus 5% Percoll, 0.01% digitonin, and a cocktail of protease inhibitors: 10 μM aprotinin, 10 μM pepstatin A, 10 μM leupeptin, 25 μM calpain inhibitor I, 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.4] containing 0.1 mM dithiothreitol (DTT) (Bettaieb and Averill-Bates, 2005). Membranes were broken using a dounce homogenizer (200 strokes/sample). After 30 min incubation on ice, debris and unbroken cells were removed by centrifugation (500 x g, 10 min) and then supernatants were centrifuged (2,500 x g, 5 min) to separate nuclei (pellet). The purity of nuclear fractions (89%) was confirmed by Western blotting using lamin A (Calbiochem, La Jolla, CA, USA) (data not shown).

2.7. Measurement of caspase and calpain activities

Cells were lysed in 20 mM piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES), 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propane-sulfonic acid (CHAPS), 10% sucrose, pH 7.2). Activities of calpain, caspase 3, caspase 4 and caspase 7 were determined using their fluorogenic substrates: Suc-LY-AM, Ac-DEVD-AMC, Ac-LEVD-AFC, and MCA-VDQVDGWK(DNP)-NH_2 (Calbiochem), respectively, by spectrofluorometry [29,30]. Activities of proteases are represented as relative cumulative fluorescence of the kinetic reaction and compared to untreated non-thermotolerant control cells (Normal).

2.8. Intracellular Free Ca^{2+} measurement

The intracellular Ca^{2+} transients were monitored using the Ca^{2+}-sensitive fluorescent indicator, Fluo 3-AM as previously described [31] with modifications. Briefly, where indicated, cells were pre-incubated with calcium chelator BAPTA-AM (100 μM) for 2 in a serum-free medium. Cells were then heated for 1 h at 42 and 43 °C. Forty five minutes before the end of the heat shock exposure, Fluo 3-AM (5 μM) was added to the cells. Cells were then washed and resuspended in PBS supplemented with PI (25 μM). Fluo 3-AM and PI fluorescence were analyzed using a FACScan cytometer (FACScan, BD Biosciences, Mississauga, ON, Canada) equipped with an argon ion laser emitting at 488 nm and a fluorescence detector band pass filter of 530/30 (for Fluo 3-AM) and 682/33 (for PI). At least 10,000 live cells were counted for each sample. WinMDI software was used to measure fluorescence intensity.

2.9. Statistical analyses

Data are expressed as means + standard error of the mean (SEM). Statistical analyses were performed using JMP program (SAS Institute).

Table 1
Primer sequences used for real time PCR to determine mRNA expression of ER stress markers.

Gene Name	Forward primer (5′–3′)	Reverse primer (5′–3′)
ATF4	GGGTTCTCCAGCGACAAGGCTAAG	AACAGGGCATCCAAGTCGAACTC
ATF6	ATGTCTCCCCTTTCCTTATATGGT	AAGGCTTGGGCTGAATTGAA
BiP	TGCTTGATGTATGTCCCCTTA	CCTTGTCTTCAGCTGTCACT
CHOP	CATCACCACACCTGAAAGCA	TCAGCTGCCATCTCTGCA
eIF2α	GAAGAGTGTGTTGGGCAGGT	TGGCTAGCAATCATGGCACT
GADD34	TCCTCTGGCAATCCCCCATA	TGGTTTTCAGCCCCAGTGTT
IRE	GCAGCAGACTTTGTCATCGG	GTGATCACACACTCCCCCTTGT
PERK	TGTCGCCAATGGGATAGTGACGAA	AATCCGGCTCTCGTTTCCATGTCT
TBP	TATAATCCCAAGCGGTTTGC	GCTGGAAAACCCAACTTCTG
XBP1	TGCTGAGTCCGCAGCAGGTG	GCTGGCAGGCTCTGGGGAAG

Comparisons between treatments were performed using one-way ANOVA and the Bonferroni-Holmes adjustment was used to control for the Family-wise error rate at a desired level ($\alpha = 5\%$). Software used was JMP Statistical Discovery 4.0 (SAS Institute Inc., Cary, NC). For significant differences, a symbol (such as *) indicates $P < 0.05$, whereas a duplicate symbol (such as **) indicates $P < 0.01$.

3. Results

3.1. Mild thermotolerance induced at 40 °C protects HeLa cells against hyperthermia-induced cytotoxicity

Mild thermotolerance was developed by heating cells for 3 h at 40 °C, whereas controls were incubated for 3 h at 37 °C. Thermotolerant cells were more resistant to cytotoxicity caused by subsequent exposure to lethal heat shock for up to 180 min at 43 °C, compared to controls (Fig. 1).

3.2. Hyperthermia (42–43 °C) activates the ER stress response in HeLa cells

The expression of several ER stress markers and the activation of key components of the ER stress signaling pathways by lethal hyperthermia were evaluated in normal non-thermotolerant HeLa cells. Immunoblot analysis showed that phosphorylation of PERK (Thr980) and its downstream target eIF2α (Ser51) were significantly increased upon exposure to lethal hyperthermia (42 - 43 °C) for 2 h, compared to control cells (37 °C) (Fig. 2A). Additionally, Ire1α phosphorylation at Ser724 increased at 42 and 43 °C compared to controls (37 °C). Phosphorylation of PERK and IRE1α, and cleavage of ATF6 (cATF6) were increased after 1 h at 43 °C (data not shown). Protein and mRNA levels of PERK, eIF2α and Ire1α were comparable in heat-shocked and control cells (Fig. 2A and B). Similarly, the cleaved form of ATF6 protein (Fig. 2A) and mRNA levels of *ATF6* (Fig. 2B) were significantly higher in cells upon thermal stress. Moreover, elevated temperatures also increased mRNA levels of the eIF2α downstream target *ATF4*, while *GADD34* levels decreased (Fig. 2B). Heat shock at 42 and 43 °C also significantly increased total protein and mRNA levels of spliced XBP1, a downstream target of IRE1α, as well as the ER chaperone BiP (Fig. 2A and B).

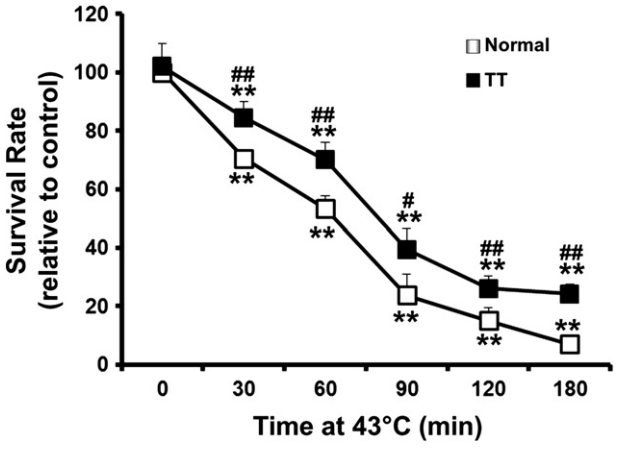

Fig. 1. Mild thermotolerance induced at 40 °C protects HeLa cells against hyperthermia-induced cytotoxicity. Normal and thermotolerant (TT) cells (5×10^6/ml) were incubated at 43 °C for up to 180 min in PBS – 1% BSA-10 mM glucose. The relative plating efficiency of each cell type was calculated by dividing the absorbance measured for a given time by the value measured for the normal control cells incubated at 37 °C for 180 min. Data (mean ± SEM, n ≥ 3) are expressed as percentage of cell survival relative to controls. (*: $P < 0.05$; **: $P < 0.01$) indicate significant difference between the hyperthermia-treated versus control cells (37 °C for 180 min) for each cell type. (#: $P < 0.05$; ##: $P < 0.01$) indicate significant difference between normal and TT cells for the indicated treatment time.

3.3. Mild thermotolerance at 40 °C mitigates hyperthermia-induced ER stress in HeLa cells

The effects of mild thermotolerance induced at fever range temperature (40 °C) on ER stress signaling were evaluated. The induction of ER stress markers in thermotolerant cells by subsequent exposure to elevated temperatures of 42 and 43 °C was significantly reduced (Fig. 2A). Indeed, mild thermotolerance mitigated hyperthermia-induced PERK (Thr980), eIF2α (Ser51) and Ire1α (Ser727) phosphorylation and decreased sXBP1 and cleaved ATF6 expression when compared to non-thermotolerant control cells (Fig. 2A). In accordance with these biochemical findings, mild thermotolerance alleviated hyperthermia-induced increases in *BiP*, *sXBP1*, *ATF4* and *ATF6* mRNA expression (Fig. 2B). *GADD34* mRNA levels were higher at 37 °C in thermotolerant cells and remained elevated upon exposure to 42 and 43 °C (Fig. 2B). The function of GADD34 is the recovery from the shutdown of protein synthesis induced by ER stress [32].

3.4. Hyperthermia (42–43 °C) induces ER stress-mediated apoptosis in HeLa cells: protective effect of mild thermotolerance at 40 °C

Next we assessed the role of the ER in hyperthermia-induced apoptosis. CHOP is known to be an important component in ER stress-mediated apoptosis [33]. Hyperthermia (42–43 °C) caused a significant increase in mRNA and protein levels of CHOP (Fig. 3A and B). There was a corresponding increase in the nuclear translocation of CHOP (Fig. 3C). Mild thermotolerance (40 °C) significantly decreased the induction of CHOP by lethal hyperthermia at both the mRNA and protein levels (Fig. 3A and B), as well as its nuclear expression (Fig. 3C).

3.4.1. Heat shock (42–43 °C) activates the calpain-calpastatin proteolytic system: attenuation by mild thermotolerance at 40 °C

Alterations in Ca^{2+} homeostasis appear to play a role in ER stress-associated apoptosis [24]. Rising temperatures from 37 °C to 43 °C caused a significant increase in intracellular free calcium levels that were detected by increased Fluo-3 fluorescence (Fig. 4A and B). Alterations in free calcium levels can activate the calcium-dependent proteolytic system calpain-calpastatin [34]. In accordance, the calpain inhibitor calpastatin underwent cleavage at 42 to 43 °C, while calpain was cleaved to generate its active form (Fig. 4C), which was confirmed by increased enzymatic activity at 42 and 43 °C, compared to 37 °C (Fig. 4D). Vinculin, a cytoskeletal protein that is a calpain substrate [35], underwent cleavage at 42 and 43 °C (Fig. 4C). The hyperthermia-induced increase in calpain activity was inhibited by BAPTA-AM, whereas vinculin cleavage was inhibited by a calpain inhibitor (data not shown). Nevertheless, thermotolerance induced at 40 °C significantly inhibited hyperthermia-induced alterations in calcium levels (Fig. 4A and B), as well as activation of the calpain-calpastatin proteolytic system, compared to normal non-thermotolerant cells (Fig. 4C and D).

3.4.2. Mild thermotolerance at 40 °C diminishes ER stress-mediated caspase activation by heat shock

Next, we investigated the role of ER-mediated caspase activation in hyperthermia-induced apoptosis in HeLa cells. The cleavage and activation of ER initiator caspase 4 were examined. Procaspase 4 is localized at the cytosolic side of the ER membrane [36] and can be activated by several mechanisms, including cleavage by calpain and by caspase 7 [37,38] and by the Ask1/IRE1α/TRAF2 complex [24,26]. The exposure of cells to lethal hyperthermia (42–43 °C) for 3 h caused the cleavage of procaspase 4 and procaspase 7 (Fig. 5A), as well increases in their enzymatic activities (Fig. 5B). Caspase 4 activation by heat was inhibited by BAPTA-AM and by a calpain inhibitor, but not by a caspase 7 inhibitor (Fig. 5B). The immunoprecipitation of IRE1α and Western blotting for Ask1, pro-caspase 4 and TRAF2 showed that the Ask1/IRE1α/TRAF2 complex was not involved in the activation of caspase 4 at 42 and 43 °C (data not shown). Once activated, caspase 4 can directly activate

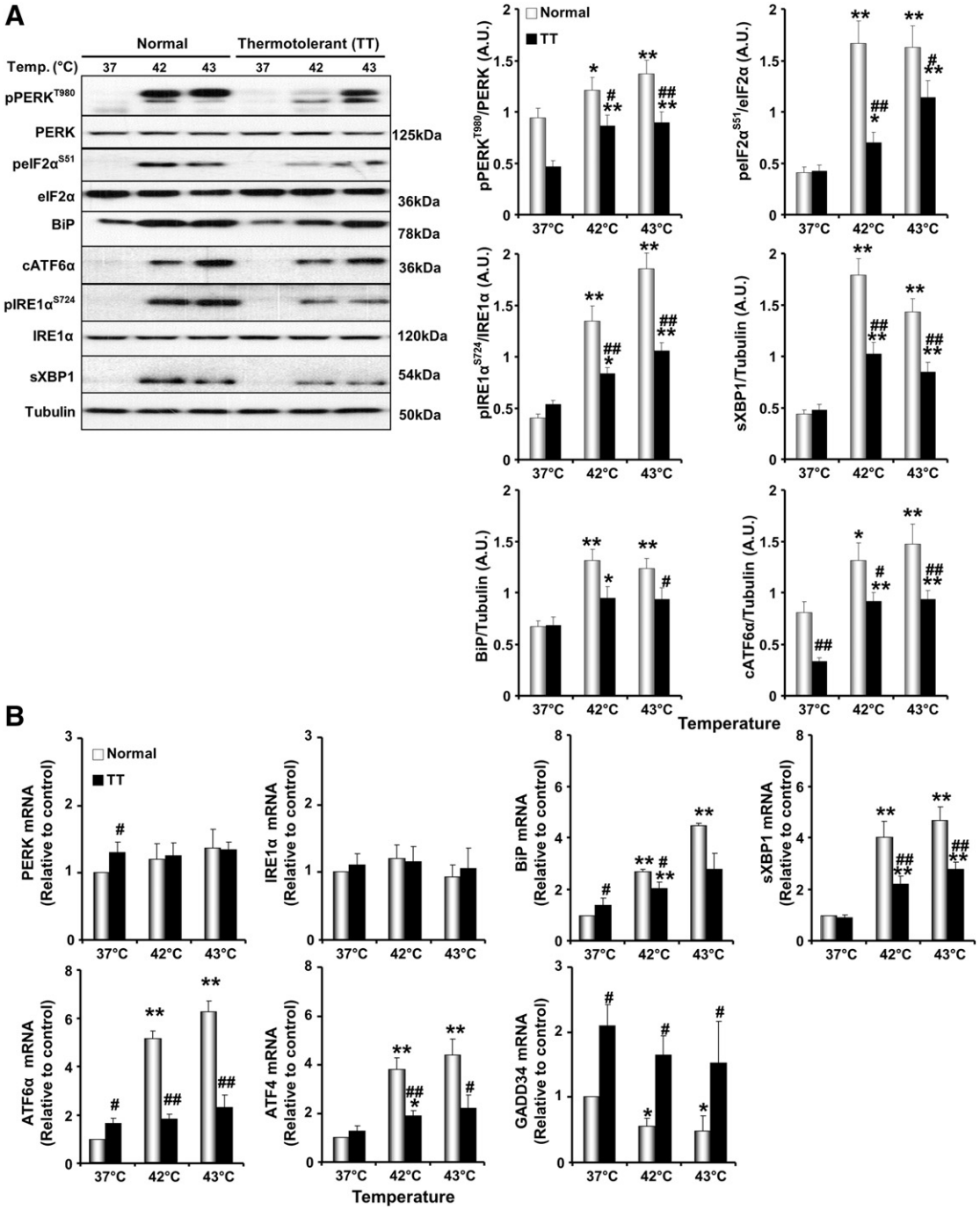

Fig. 2. Thermotolerance induced by mild hyperthermia at 40 °C alleviates heat shock-induced ER stress. Normal and thermotolerant (TT) HeLa cells were incubated at 37, 42 and 43 °C for 2 h. A) Lysates were immunoblotted for pPERK (Thr980), PERK, peIF2α (Ser51), eIF2α, BiP, cleaved ATF6 (cATF6α), pIRE1α (Ser724), IRE1α, sXBP1 and tubulin. Bar graphs represent normalized data expressed as arbitrary units (A.U.) for pPERK/PERK, peIF2α/eIF2α, pIRE1α/IRE1α, sXBP1/tubulin, BiP/tubulin and cATF6α/tubulin from at least 3 independent experiments and presented as means ± SEM. B) *PERK*, *IRE1α*, *BiP*, *sXBP1*, *ATF6α*, *ATF4* and *GADD34* mRNA levels were measured by quantitative real-time PCR and normalized against the housekeeping gene TATA-box binding protein (TBP). Data represent means ± SEM of 4 independent experiments. A and B. (*; $P < 0.05$, **; $P < 0.01$) indicates significant difference between 42 and 43 °C-treated versus 37 °C-treated cells, and (#; $P < 0.05$, ##; $P < 0.01$) indicates significant difference between thermotolerant versus normal cells incubated at the same temperature.

caspase 9, which in turn catalyzes the cleavage of procaspase 3 [37,39,40]. The downstream procaspase 3 also underwent cleavage as well as an increase in enzymatic activity at 42–43 °C, compared to controls at 37 °C (Fig. 5A and B). Hyperthermia-induced procaspase cleavage and activation of caspases 4, 7 and 3 were significantly attenuated in mild thermotolerant (40 °C) cells (Fig. 5A and B).

The subsequent step was to determine the role of ER stress-induced apoptosis in the downstream execution phase of apoptosis. This was carried out using inhibitors of calpain and caspase proteases. Caspase 3 activation was significantly diminished by a caspase 4 inhibitor (Fig. 5B). This confirms that caspase 4 plays a role in caspase 3 activation by lethal hyperthermia through the ER, which is independent of the mitochondrial pathway. However, the caspase 4 inhibitor only partially inhibited caspase 3 activation, which indicates that hyperthermia can activate apoptosis simultaneously by other pathways. Caspase 3 has been implicated in nuclear chromatin condensation and DNA fragmentation through activation of caspase activated DNase (CAD) and the cleavage of poly(ADP-ribose) [41]. Hyperthermia (42–43 °C) caused

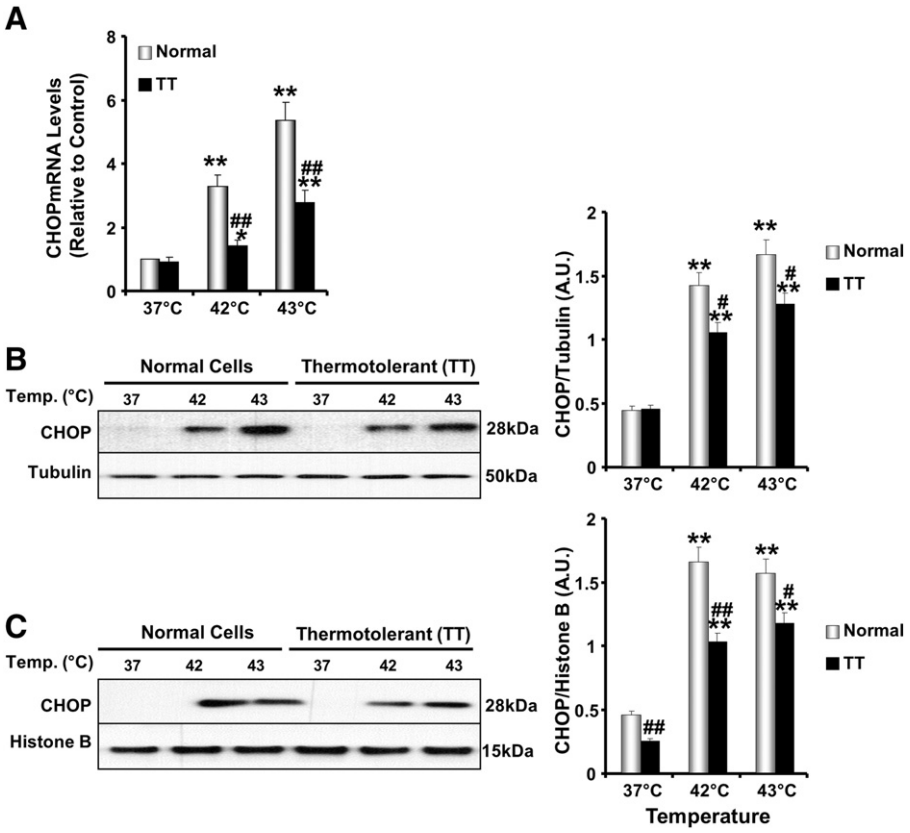

Fig. 3. Mild thermotolerance at 40 °C decreases heat shock-induced CHOP expression and nuclear translocation. Normal and thermotolerant HeLa cells were incubated at 37, 42 and 43 °C for 3 h. A) CHOP mRNA levels were measured by quantitative real-time PCR and normalized against TATA box binding protein (TBP) as housekeeping gene. Data represent means ± SEM of 4 independent experiments. Total (B) and nuclear (C) protein levels of CHOP. Bar graphs represent normalized data expressed as arbitrary units (A.U.) for CHOP/tubulin (B) or CHOP/histone B (C) from at least 3 independent experiments and presented as means ± SEM. (*; $P < 0.05$, **; $P < 0.01$) indicates significant difference between 42 and 43 °C-treated versus 37 °C-treated cells, and (#; $P < 0.05$, ##; $P < 0.01$) indicates significant difference between thermotolerant versus normal cells incubated at the same temperature.

chromatin condensation in cells, relative to controls at 37 °C (Fig. 5C and D). Mild thermotolerance at 40 °C decreased the percentage of apoptotic cells at 42 °C and 43 °C (Fig. 5C and D). Interestingly, hyperthermia-induced chromatin condensation was inhibited significantly by a calcium chelator BAPTA-AM and by a caspase 4 inhibitor, but not by a calpain inhibitor (Fig. 5C and D). Apoptosis was only partially inhibited by BAPTA-AM and the caspase 4 inhibitor. Propidium iodide was used to distinguish between cell death by apoptosis and necrosis. The percentages of necrotic cells at 42 and 43 °C were much lower than those of apoptotic cells (Fig. 5E), and they were not altered by BAPTA-AM or the caspase 4 and calpain inhibitors.

3.5. Hsp72 plays a role in the protective effect of mild thermotolerance against activation of ER stress and apoptosis by hyperthermia

The development of thermotolerance by exposure of HeLa cells to mild hyperthermia (40 °C) for 3 to 24 h led to the accumulation of several Hsps [29,30]. Among them, Hsp72 protected rat pheochromocytoma PC12 cells from apoptosis induced by the ER stressors thapsigargin and tunicamycin [42]. To investigate the molecular mechanisms underlying regulation of ER stress and ER stress-mediated apoptosis by mild thermotolerance (40 °C), we determined the effects of Hsp72 deficiency on these processes. HeLa cells with stable knockdown (KD) of Hsp72 (sh-Hsp72) and control cells using sh-scramble (sh-SCR) were generated using lentiviral shRNA. Immunoblot analysis demonstrated a significant decrease in Hsp72 expression by about 40.3% in knockdown cells at 37 °C (Fig. 6A, white bars). Furthermore, there was no increase in Hsp72 in thermotolerant KD cells (TT + sh-Hsp72) as compared to thermotolerant WT cells (TT + sh-SCR) (Fig. 6A). The total Hsp72 (inducible + constitutive) expression in thermotolerant KD cells after 24 h was only 16.7% compared to thermotolerant cells (black bars: TT + sh-SCR versus TT + sh-Hsp72).

Hyperthermia (42–43 °C for 2 h) increased ER stress in normal (non-thermotolerant, 3 h at 37 °C) cells (sh-SCR) that was diminished in thermotolerant (3 h at 40 °C) (TT + sh-SCR control) cells (Fig. 6B). To determine the role of Hsp72 in the protective effects of mild thermotolerance at 40 °C, we compared the magnitude of the induction of ER stress by hyperthermia in thermotolerant (TT + sh-SCR control) and thermotolerant Hsp72 KD (TT + sh-Hsp72) cells. Hsp72 deficiency in thermotolerant cells increased ER stress and counteracted the protective effects of mild hyperthermia (40 °C) as evidenced by increased phosphorylation of PERK (Thr^{980}), eIF2α (Ser^{51}) and IRE1α (Ser^{724}), and increased expression of sXBP1 (Fig. 6B). Additionally, Hsp72 KD alleviated the protective effects of mild thermotolerance against the induction of ER stress-mediated apoptosis by hyperthermia (42–43 °C) and sensitized thermotolerant KD cells to the activation of caspases 4, 7 and 3 (Fig. 6C). Together, these data indicate that Hsp72 plays an important role in mediating the protective effects of mild thermotolerance (40 °C) against ER stress-mediated apoptosis triggered by lethal hyperthermia at 42–43 °C.

4. Discussion

This study shows that the exposure of human cervical cancer cells to lethal hyperthermia at 42 to 43 °C for 2 h activated the 3 branches of the ER stress response; there was increased phosphorylation of PERK, eIF2α and IRE1α, cleavage of ATF6, and increased expression of sXBP1. When HeLa cells were exposed to lethal hyperthermia for a longer time of 3 h, they underwent apoptosis through the ER. This involved the induction of CHOP, alterations in calcium levels and the activation of ER proteases:

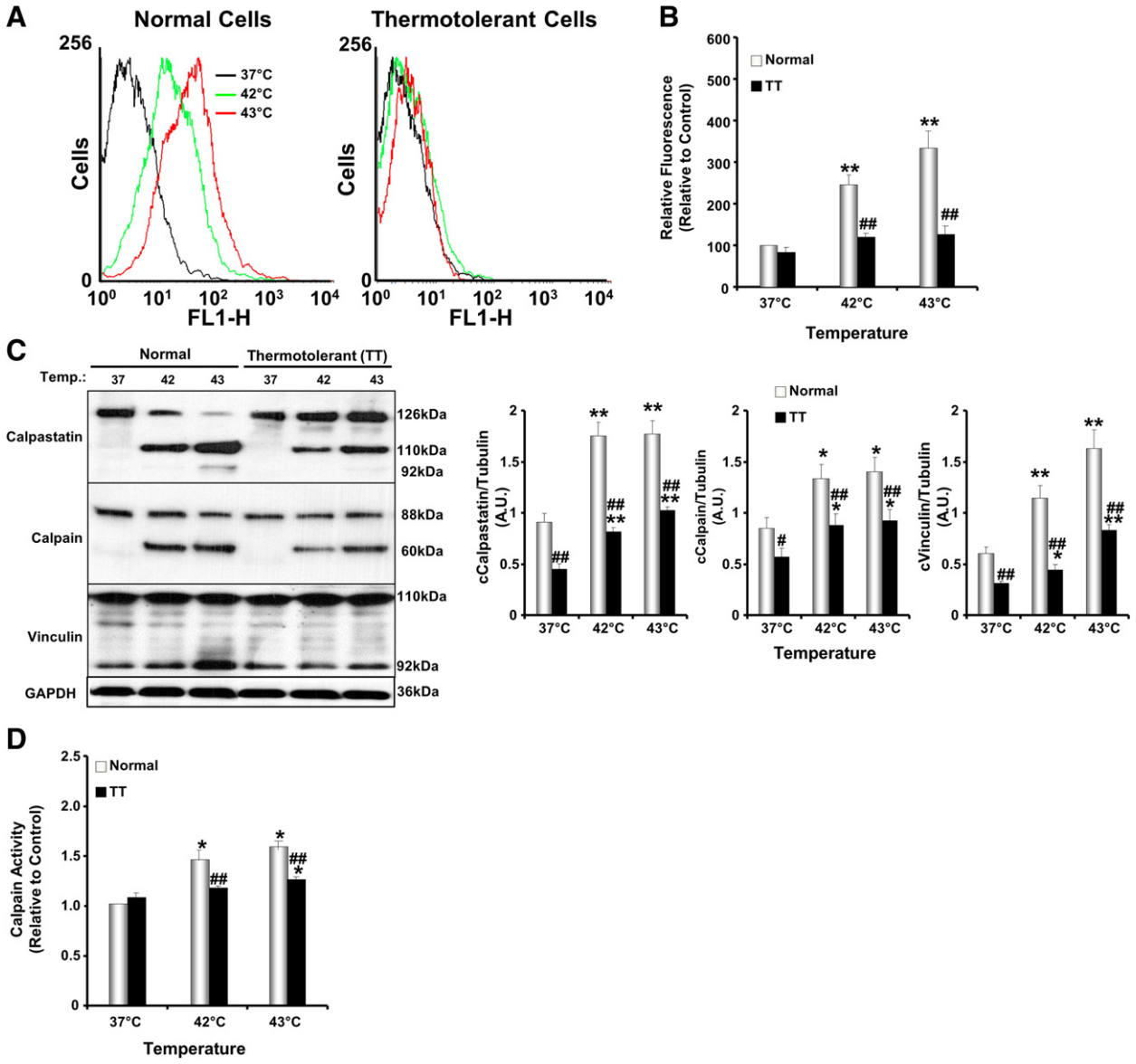

Fig. 4. Mild thermotolerance protects against heat shock-induced calcium release and activation of calcium-dependent proteases. A) Normal and thermotolerant HeLa cells were incubated at 37, 42 and 43 °C for 1 h and then loaded with Fluo 3-AM for 20 min. Histograms represent relative levels of cytoplasmic Ca^{2+}. B) Bar graph represents green fluorescence (FL-1) that was quantified using WinMDI software and normalized to normal cells incubated at 37 °C (100%) and presented as means ± SEM ($n \geq 3$). C) Immunoblots of calpastatin, calpain and vinculin in lysates of normal and thermotolerant cells that were incubated at 37, 42 and 43 °C for 3 h. Bar graphs represent normalized data for cleaved (c) forms of each protein, presented as means ± SEM ($n \geq 3$). D) Enzymatic calpain activity in normal and thermotolerant HeLa cells incubated at 37, 42 and 43 °C for 3 h. Bar graph represents normalized data presented as means ± SEM. B, C and D. (*; $P < 0.05$, **; $P < 0.01$) indicates significant difference between 42 and 43 treated versus 37 °C treated cells, and (#; $P < 0.05$, ##; $P < 0.01$) indicates significant difference between thermotolerant versus normal cells incubated at the same temperature.

the calpain-calpastatin proteolytic system and caspase 4. On the other hand, mild thermotolerance induced by pre-conditioning of HeLa cells at a non-lethal temperature of 40 °C diminished activation of the ER stress response and protected cells against apoptosis induced by lethal doses of hyperthermia (42–43 °C). These protective effects of mild thermotolerance were mediated at least in part through the induction of Hsp72. shRNA mediated KD of Hsp72 mitigated the protective effects of mild thermotolerance against the induction of ER stress and ER stress-mediated apoptosis by lethal hyperthermia. Together, these findings reveal an important role for Hsp72 in mediating the adaptive survival response induced by heat preconditioning of tumor cells at a low dose, fever temperature of 40 °C.

Hyperthermia has been shown to activate the ER stress response in several cell types including cancer cells. An *in vivo* study showed that hyperthermia (45 °C for 50 min) activated ER stress markers (XBP1 splicing and eIF2α phosphorylation) in the rat cortex [43]. In human glioma A172 cells, exposure to heat at 40.5, 42 or 43.5 °C for 40 min induced ER stress through increased eIF2α phosphorylation and XBP1 splicing resulting in the induction of *GADD34, CHOP* and *BiP* gene expression [43]. We obtained similar ER stress responses in hyperthermia-treated HeLa cells, although the 3 branches, PERK/eIF2α, ATF6 and IRE1α/XBP1, were activated. Conversely, hyperthermia (43 °C for 3 to 12 h) inhibited the induction of ER stress response genes in AD293 cells, whereas several ER stress genes including *BiP, CHOP* and *GADD34* as well as XBP1 splicing were induced by mild hyperthermia (5 h at 40 °C) [44]. These findings seem to be specific to AD293 cells since responses were low or absent in hepatic cells, insulin-secreting cells and mouse embryonic fibroblasts [44]. Findings for heat–induced ER stress responses seem to vary between different cell and tissue types and warrant additional investigation.

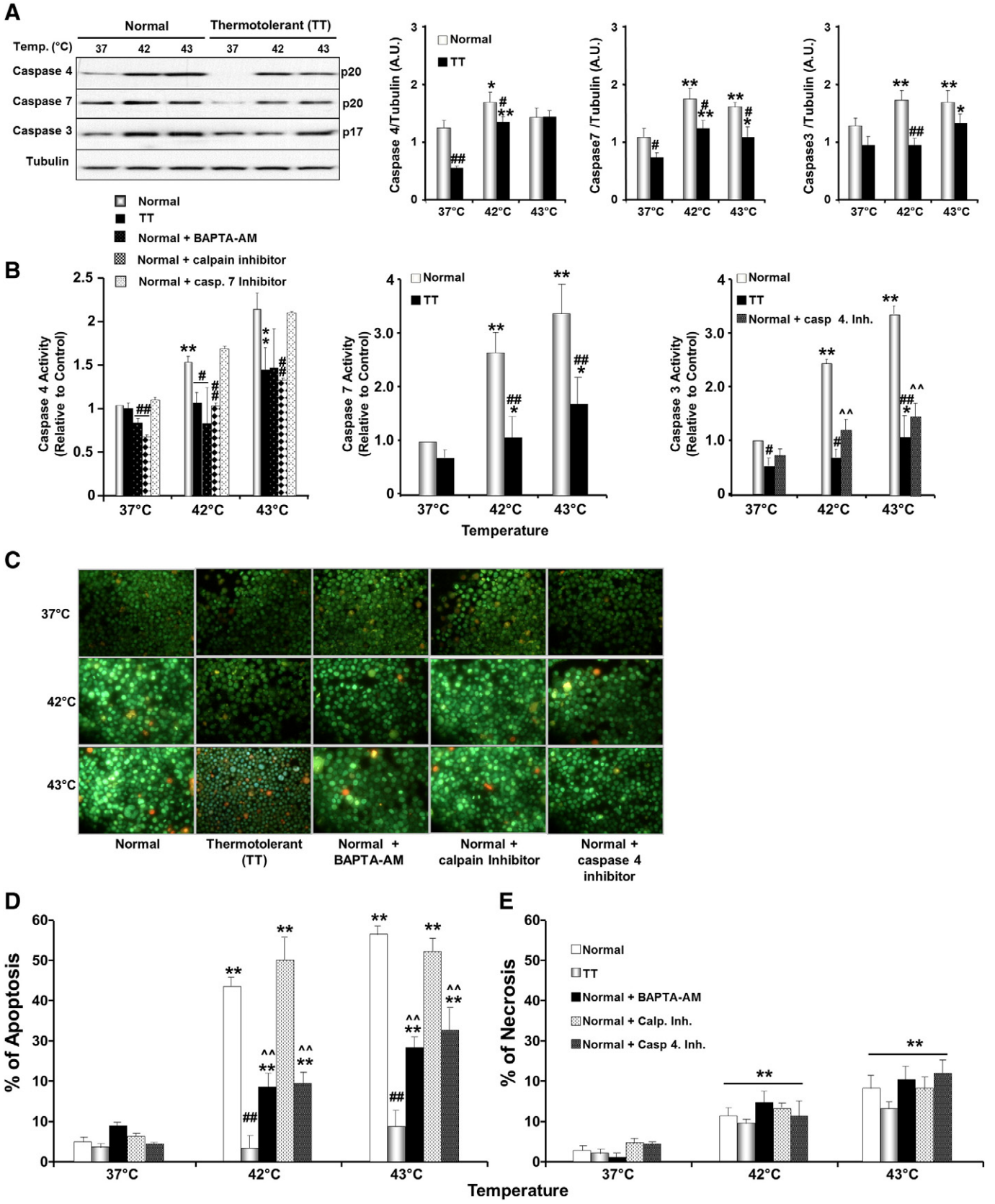

Fig. 5. Mild thermotolerance at 40 °C protects cells against heat shock-induced apoptosis. Normal and thermotolerant HeLa cells incubated at 37, 42 and 43 °C for 3 h. A) Immunoblots of cleaved forms of caspases 4, 7 and 3 in cell lysates. Bar graphs represent normalized data presented as means ± SEM from 4 independent experiments. B) Enzymatic activity of caspases 4, 7 and 3 in cell lysates. For caspase 3 activity, cells were pretreated with a caspase 4 inhibitor. Bar graphs represent normalized data presented as means ± SEM. C) Normal cells were pretreated with the indicated inhibitor as described in the Methods section. Cells were stained for apoptosis (Hoechst 33258, green) and necrosis (PI, red). Bar graphs represent apoptosis (D) and necrosis (E) levels and presented as means ± SEM from at least 3 independent experiments. A, B and D. (*; $P < 0.05$, **; $P < 0.01$) indicates significant difference between 42 and 43 treated versus 37 °C treated cells, and (#; $P < 0.05$, ##; $P < 0.01$) indicates significant difference between indicated conditions versus normal cells incubated at the same temperature. (^; $P < 0.05$, ^^; $P < 0.01$) indicates significant difference with and without inhibitor.

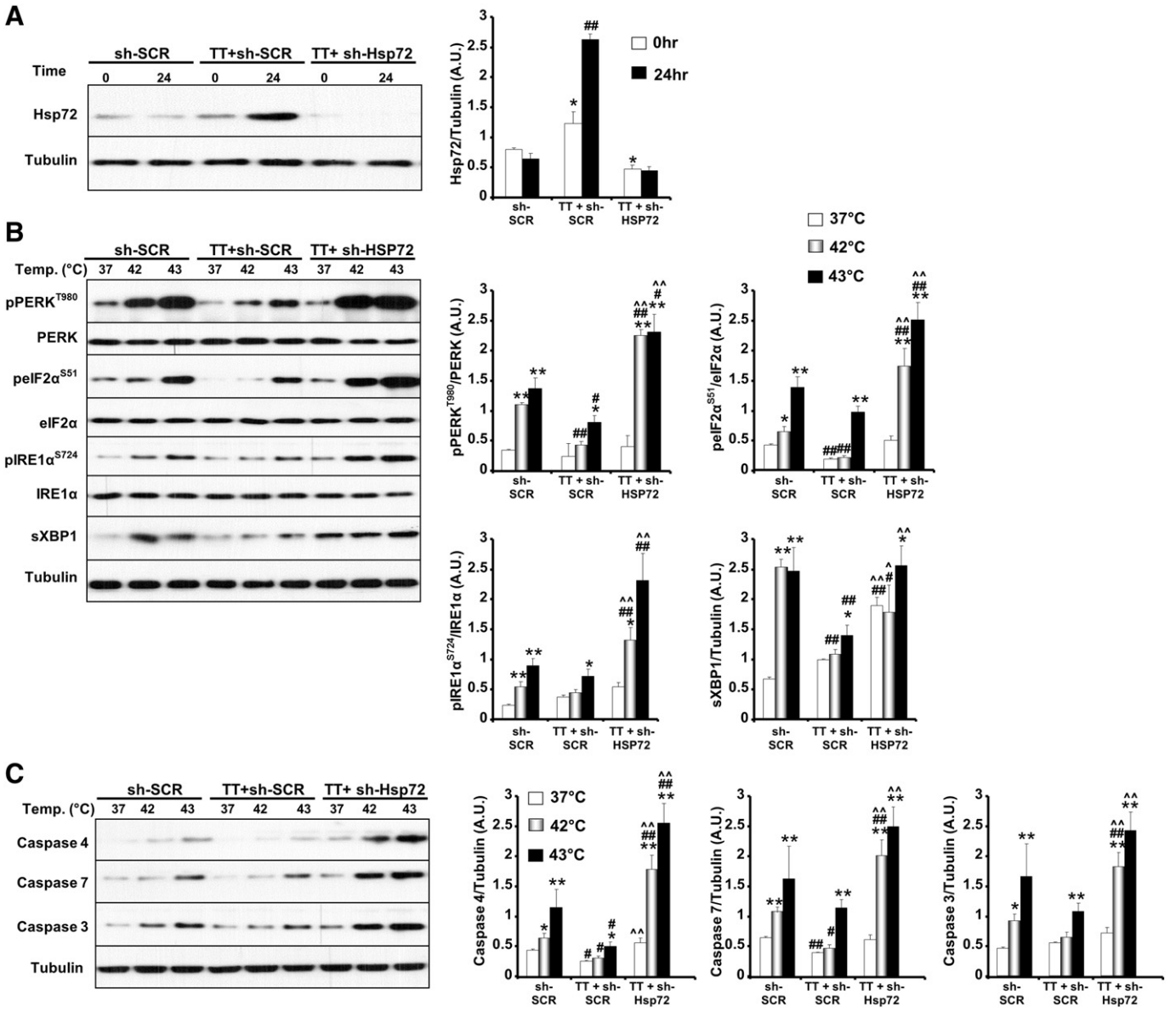

Fig. 6. Hsp72 deficiency mitigates heat shock-induced ER stress and caspase activation. A) Immunoblots of Hsp72 expression in normal non-thermotolerant and thermotolerant (TT) HeLa cells expressing a scrambled (SCR) control-shRNA or shRNA targeted to Hsp72 (sh-Hsp72) upon 3 h exposure to 40 °C followed by 0 h or 24 h recovery at 37 °C. Bar graphs represent normalized data expressed as arbitrary units (A.U.) for Hsp72/tubulin from 3 independent experiments. (*) indicates significant difference between cells incubated at 42 or 43 °C versus cells incubated at 37 °C. B) Lysates from normal and thermotolerant cells expressing shRNA targeted to Hsp72 (sh-Hsp72) or a scrambled control-shRNA and exposed for 2 h to 42 or 43 °C were immunoblotted for pPERK (Thr980), PERK, peIF2α (Ser51), eIF2α, BiP, pIRE1α (Ser724), IRE1α, spliced XBP1 (sXBP1), and tubulin. Bar graphs represent normalized data expressed as arbitrary units (A.U.) for pPERK/PERK, peIF2α/eIF2α, BiP/tubulin, pIRE1α/IRE1α and sXBP1/tubulin from 3 independent experiments and presented as means ± SEM. C) Lysates from normal and thermotolerant cells expressing sh-Hsp72 or a scrambled control-shRNA and exposed for 2 h to 42 or 43 °C were immunoblotted for active caspases 4, 7 and 3. B and C. (*; $P < 0.05$, **; $P < 0.01$) indicates significant difference between 42 and 43 °C versus 37 °C for each cell type. (#; $P < 0.05$, ##; $P < 0.01$) indicates significant difference between indicated cell type and sh-SCR for the same temperature. (^; $P < 0.05$, ^^; $P < 0.01$) indicates significant difference between TT + sh-Hsp72 and TT + sh-SCR cells.

Apoptosis and necrosis are the best described mechanisms by which different stresses can cause cell death. The induction of apoptosis and necrosis by hyperthermia is dependent on the dose and duration of heat stress [30]. Hyperthermia (41–45 °C) can induce apoptosis through the death receptor, mitochondrial [8,29,30,45–47] and ER pathways [43,44,48]. The mechanisms through which lethal hyperthermia causes ER stress-induced cell death are poorly understood. In melanoma and non-melanoma skin cancer cells, hyperthermia caused ER stress-mediated apoptosis that involved caspase 4/12 [48]. Our current study provides new insights into the molecular mechanisms underlying ER stress-mediated apoptosis in HeLa cells. Hyperthermia (42–43 °C) increased the expression and nuclear translocation of the pro-apoptotic transcription factor CHOP. The activation of PERK and ATF6 leads to transcriptional induction of CHOP, which appears to mediate apoptosis by up-regulating the expression of genes such as *GADD34* and BH3-only pro-apoptotic proteins *Bim*, *PUMA* and *Bax* and/or by inhibiting gene expression of *Bcl-2* [49]. Hyperthermia is known to alter the permeability of plasma membranes resulting in a calcium spike [10]. We showed that ER stress-induced apoptosis was mediated by the calcium-dependent proteolytic system calpain-calpastatin, which resulted in calpain-dependent activation of the ER resident caspase 4. Furthermore, caspase 4 played a role in caspase 3 activation and DNA fragmentation by lethal hyperthermia at 42 to 43 °C.

Thermotolerance is an adaptive survival response induced by low dose heat preconditioning in which cells become resistant to exposure to a subsequent toxic stress such as heat shock, oxidative stress or environmental toxins [29,37,50,51]. Adaptive survival responses allow cells and organisms to continue their normal functions despite exposure to

an adverse stimulus. These responses involve a variety of survival strategies that appear to be mediated by a group of anti-apoptotic genes and their products (e.g. Hsps, antioxidants), which protect cells against diverse toxic and environmental stresses [52]. If the adaptive survival response cannot protect the cell against a toxic stress exposure, then the damaged cell will be removed by death processes such as apoptosis and/or necrosis. This study shows that mild thermotolerance induced by low dose hyperthermia (40 °C) diminished the activation of ER stress markers by subsequent lethal heat shock at 42–43 °C in HeLa cells. In addition, mild thermotolerance (40 °C) protected cells against lethal hyperthermia-induced pro-apoptotic events including the disruption of calcium homeostasis, the activation of calpain and caspase 4, and nuclear chromatin condensation.

It is worth noting that Hsp72, the stress inducible form of cytosolic Hsp70, inhibits several features of the intrinsic and extrinsic apoptotic pathways [45–47,53]. However, the detailed molecular mechanisms by which Hsp72 inhibits ER stress and apoptosis through the ER are not entirely clear. This study shows that Hsp72 deficiency alleviated the protective effects of mild thermotolerance (40 °C) against hyperthermia in HeLa cells. This suggests that Hsp72 is a key element that diminishes hyperthermia-induced ER stress, which occurred at the level of PERK/eIF2α, ATF6 and IRE1α/XBP1. In addition, Hsp72 deficiency abrogated the protective effect of mild thermotolerance against hyperthermia-induced apoptosis through the ER in HeLa cells. Further, Hsp72 was shown to protect PC12 cells from apoptosis caused by the ER stress inducers thapsigargin and tunicamycin [42]. The protective effect involved a direct physical interaction between Hsp72 and the cytosolic domain of IRE1α that enhanced IRE1α/XBP1 signalling at the ER, promoting adaptation to ER stress and cell survival. Hsp72 enhanced cell survival under ER stress conditions, in particular the IRE1α/XBP1 axis. The overexpression of Hsp72 decreased the induction of ER stress markers (BiP, CHOP, XBP1) by tumor necrosis factor (TNF)-α in insulinoma-derived MIN6 β-cells [54]. Moreover, other studies have shown that Hsp72 inhibits CHOP-induced apoptosis through binding to Bax and preventing its translocation to mitochondria in RAW 264.7 macrophages [55].

It appears that the harsh conditions of the tumor microenvironment such as low nutrient supply, hypoxia, low extracellular pH, nutrient deprivation and metabolic changes could lead to ER stress [56]. In tumors, the balance between the level of ER stress and the capacity of the cell to withstand toxic insults could determine cell outcome: survival versus cell death [57,58]. The ER stress response could therefore provide a survival and proliferative advantage whereas apoptosis through the ER could inhibit tumor growth. Treatments that aim to diminish the adaptation response of ER stress and increase the pro-apoptotic aspects of the ER signalling pathway could therefore be beneficial for the elimination of tumors [58,59]. In this regard, hyperthermia could be useful in cancer therapy through regulating ER stress pathways in tumor cells.

Acknowledgements

The authors thank Mr Bertrand Fournier (Service de consultation en analyse de données, Université du Québec à Montréal) for statistical analyses, Mr Denis Flipo (MSc) for assistance with FACscan analyses, and NSERC Canada for financial support (DAB). AB receives research support from NIDDK (K99DK100736).

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