Activation of ER stress and apoptosis by hydrogen peroxide in HeLa cells: Protective role of mild heat preconditioning at 40°C

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Abstract
The accumulation of misfolded proteins in the endoplasmic reticulum (ER) during stress conditions causes activation of the unfolded protein response (UPR). This study determines whether thermotolerance developed at a mild temperature (40°C) can alter induction of ER-mediated stress and apoptosis by H2O2 in HeLa cells. Protein expression of PERK, p-PERK, eIF2α and p-eIF2α was increased in thermotolerant compared to non-thermotolerant cells. Thus, mild thermotolerance enhanced pro-survival effects of the PERK/eIF2α branch of the UPR. A short exposure (15 min) of cells to H2O2 (15–50 μM) activated the UPR: expression of p-PERK, p-eIF2α and p-IRE1α increased, and ATF6 cleavage occurred. Longer exposure (1–3 h) to H2O2 induced ER-mediated apoptosis, whereby CHOP expression increased, and enzymatic activity of calpain, caspase-7,-12 and -9 also increased. These pro-apoptotic events and clonogenic cell killing were all diminished in thermotolerant cells. Activation of caspases-4/-12 was decreased by the calcium chelator BAPTA-AM, and by inhibitors of calpain and caspase-7, confirming the roles of calcium, calpain and caspase-7 in activation of ER-mediated apoptosis by H2O2. In thermotolerant cells with decreased levels of PERK by siRNA, there was partial reversal of resistance to H2O2-induced apoptosis. Hence, a causal connection exists between the ER stress response and resistance to H2O2-induced apoptosis. Mild thermotolerance plays a protective, anti-apoptotic role by increasing the threshold for induction of ER-mediated apoptosis by H2O2. Moreover, the adaptive response (UPR) dominates during milder H2O2 stress, whereas ER-mediated apoptosis occurs during more severe stress.

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1. Introduction
Reactive oxygen species (ROS) such as superoxide and hydrogen peroxide (H2O2) are recognized as critical signaling molecules that take part in different cellular functions such as development, proliferation and differentiation [1]. ROS can trigger cellular dysfunction by damaging bio-molecules such as lipids, proteins, carbohydrates and nucleic acids [2]. Levels of ROS are tightly controlled by scavenging enzymes (e.g. catalase and superoxide dismutase (SOD)) and antioxidants (e.g. glutathione and vitamin E) that can detoxify them. When levels of pro-oxidants overwhelm the antioxidant defenses, disturbance of the redox equilibrium occurs, resulting in oxidative stress. This can lead to the activation of stress signaling pathways and transcription factors [1,3]. Although the molecular mechanisms by which ROS activate these pathways are unclear, their activation can lead to different consequences, including growth arrest, senescence, up-regulation of death proteins, and cell death by apoptosis or necrosis.

Cells undergo apoptosis by three pathways involving death receptors, mitochondria and the endoplasmic reticulum (ER). Protein chaperones such as Bip/glucose-related protein 78 (GRP78), GRP94, calnexin and protein disulphide isomerase (PDI) assure correct folding of newly-synthesized proteins in the ER [4]. A variety of conditions including glucose deprivation, hypoxia, disturbance of calcium homeostasis, and excess ROS can perturb ER function, leading to accumulation of unfolded proteins in the ER [3,5]. This phenomenon, known as ER stress, activates signaling pathways such as the unfolded protein response (UPR) and ER-associated protein degradation (ERAD). The UPR is a survival response that aims to recover normal cellular function in the face of adverse conditions. However, if UPR activation is not able to rescue cells from ER stress and correct the defects in protein folding, then cells are generally eliminated by ER-mediated apoptosis [3,6–8]. The ER stress response involves 3 distinct mechanisms: (i) translational attenuation to decrease the synthesis of new proteins; (ii) transcriptional activation of genes for ER chaperones and ERAD molecules; and (iii) ERAD, which involves translocation of misfolded or aggregated ER proteins to the cytoplasm, where they undergo proteosomal degradation [5]. The activation of the
UPR is mediated by three distinct ER stress sensors: protein kinase RNA (PKR)-like ER kinase (PERK), activating transcription factor-6 (ATF6) and inositol-requiring protein-1 (IRE1). In non-stressed cells, these sensors are retained in the ER lumen by interactions with Bip/GRP78. During ER stress, Bip releases these three sensors, leading to their activation [5].

To counteract the multitude of processes that can trigger death, cells have developed a wide variety of survival strategies that can prevent inappropriate death [9]. The pre-exposure to sub-lethal doses of different stresses (e.g., ROS, heat shock, ischemia/reperfusion (IR)) can lead to adaptive responses that allow cells and organisms to continue normal function in the face of an adverse stimulus [9–14]. Adaptive responses appear to be mediated by a group of anti-apoptotic genes and their products (e.g., Hsps, antioxidants) that protect cells against diverse toxic and environmental stresses. If the adaptive response cannot protect the cell against an adverse exposure, then the damaged cell will be eliminated by apoptosis or necrosis [9,14]. Thermotolerance is an adaptive survival response induced by heat preconditioning whereby cells become resistant to a subsequent lethal insult such as that triggered by heat shock, ROS or environmental stressors [15,16]. This phenomenon is generally associated with the accumulation of heat shock proteins (Hsp) [15,17,18]. Thermotolerance can be developed following shorter exposures (e.g., 30 min) to lethal temperatures (42–45 °C) [15], or during continuous heating (e.g., 24 h) at non-lethal temperatures (39.5–41.5 °C). The induction of thermotolerance by lower, fever-range temperatures such as 40 °C has received little attention.

We recently reported that, besides Hsps [13], mild thermotolerance (40 °C) can induce several antioxidants in human cervical carcinoma (HeLa) cells [19]. The present work determines whether mild thermotolerance can induce other adaptive survival responses such as the UPR in HeLa cells. This study also investigates the role of the ER in H2O2-induced cell death, and whether mild thermotolerance (40 °C) can alter the induction of ER stress by H2O2 and protect cells against ER-mediated apoptosis.

2. Material and methods

2.1. Cell culture

HeLa cells (ATCC #CCL-2) were grown in monolayer in Dulbecco's modified Eagle's medium (Invitrogen Canada, Burlington, ON, Canada) containing 10% fetal bovine serum (FBS) (Invitrogen Canada), in tissue culture flasks (Sarstedt, St. Laurent, QC, Canada) at 37 °C in a humidified atmosphere of 5% CO2 in a water jacketed incubator [20]. Culture medium was replaced with fresh medium 24 h before experiments. To induce thermotolerance, 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 [10]. The cells were grown to near confluence and then harvested using 0.25% (w/v) trypsin–0.02% (w/v) EDTA solution, and washed by centrifugation (1000×g, 3 min). There was no loss of viability in cells heat-treated at 40 °C for 3 h, evaluated by trypsin blue exclusion (data not shown).

2.2. Treatment with H2O2

Cells (106/ml) were incubated with H2O2 (Sigma-Aldrich, Canada, ON, Canada) (0, 15–50 μM) in DMEM containing 10% FBS at pH 7.4 for different times ranging from 15 min to 3 h at 37 °C [19,21]. Cells were pretreated for 1 h with 50 μM calcium chelator BAPTA-AM (Sigma-Aldrich), 20 μM calpain inhibitor I (Ac-LLL-NH2) (Sigma-Aldrich), 50 μM caspase-7/3 inhibitor I (5-[(S)-[(S)-2-(methoxymethyl)pyrrolidino)sulfonilylsatin] (Calbiochem), 20 μM caspase-4 inhibitor I, (LEVD-CHO) (Calbiochem) or 10 μM caspase-12 inhibitor (Z-ATAD-fluoromethyl ketone (FMK)) (MBL International Corporation), where indicated. Cells were washed by centrifugation (1000×g, 3 min) to remove inhibitors and H2O2, and then analyzed for the UPR response and ER apoptotic signaling.

2.3. Morphological analysis of apoptosis

Thermotolerant and non-thermotolerant cells (106/ml) were labeled with Hoechst 33258 (50 μg/ml) (blue-green fluorescence) (Sigma Chemical Co.), which binds to condensed nuclear chromatin of apoptotic cells [22]. Cells were washed with PBS and then propidium iodide (PI) (50 μg/ml) was added to visualize necrotic cells (red fluorescence). Observations were made by fluorescence microscopy (model IM, Carl Zeiss Canada Ltd, St. Laurent, QC) and photographs were taken by digital camera (camera 3CCD, Sony DVC–950P, Empix imaging Inc, Mississauga, ON). Images were analyzed using Northern Eclipse software (Empix Imaging). Cells were classified using the following criteria: (1) live cells with normal nuclei, pale blue/green chromatin with organized structure; (2) apoptotic cells with bright blue/green condensed or fragmented chromatin; (3) necrotic cells (red, enlarged nuclei with smooth normal structure). Fractions of apoptotic or necrotic cells were calculated relative to total cells. For each condition, 300 cells were counted.

2.4. Clonogenic cell survival assay

Clonogenic cell survival measures the ability of cells to undergo cell proliferation following a toxic insult. Non-thermotolerant cells and thermotolerant (40 °C, 3 h) cells were exposed to H2O2 (0–150 μM) for 2 h at 37 °C in a final volume of 1.0 ml in D-MEM containing 10% FBS. After the appropriate time, the cells were washed three times by centrifugation (1000×g, 2 min) to stop the incubation [19]. The cells were resuspended in culture medium, diluted to the appropriate concentration and plated in tissue culture dishes (60 mm × 15 mm), which were incubated at 37 °C in an incubator in an atmosphere of 5% CO2 for 10 days. The dishes were then washed with PBS, fixed with 95% ethanol and stained with methylene blue before counting macroscopic colonies (>50 cells). Cytotoxicity was expressed as the mean number of colonies obtained relative to the mean number of colonies obtained in the control. Two hundred cells were seeded in the control plates, but where there was a loss of cell survival, cells were plated at several different densities to ensure that countable colonies would be obtained, and the results were corrected accordingly. We have previously demonstrated that, in this system, there is linearity between the number of cells plated and colonies formed over the range of 10–104.

2.5. Preparation of whole cell lysates

For analysis of protein expression of PERK, p-PERK, eIF2α, p-eIF2α, ATF6, Bip, IRE1α and p-IRE1α, non-thermotolerant (37 °C, 3 h) and thermotolerant (40 °C, 3 h) cells were harvested and then exposed to H2O2 for 15 min. For detection of CHOP, calpastatin, caspase-4 and caspase-12, thermotolerant and non-thermotolerant cells were harvested and incubated with H2O2 at 37 °C for 1 or 2 h. Cells were washed by centrifugation (1000×g, 3 min) in buffer A (100 mM sucrose, 1 mM EGTA, 20 mM 3-(N-morpholino)propanesulfonic acid (MOPS), pH 7.4) [23]. The supernatant was discarded, pelleted cells were resuspended in lysis buffer B [buffer A plus 5% Percoll, 0.1% digitonin and a cocktail of protease inhibitors: 10 μM aprotinin, 10 μM pepstatin A, 10 μM leupeptin, 25 μM calpain inhibitor I and 1 mM phenylmethylsulfonyl fluoride (PMSF)] and incubated on ice for 1 h. Then, the proteins of whole cell lysates were isolated in the supernatant, by a 10 min centrifugation step at 2500×g to remove nuclei and unbroken cells [20], and used for detection of proteins by Western blotting.
2.6. Preparation of subcellular fractions

Cells were treated with H_2O_2 and then subcellular fractions were prepared as described previously [19,20]. Thermotolerant and non-thermotolerant cells were washed in buffer A and resuspended in buffer B containing 0.1 mM dithiothreitol (DTT). Lysates were homogenized using a dounce homogenizer (50 strokes/sample). After a 30 min incubation on ice, unbroken cells and nuclei were pelleted by centrifugation at 2500×g for 10 min. The supernatant was centrifuged further at 15,000×g for 15 min to separate the mitochondrial fraction. The supernatant was further centrifuged at 100,000×g for 1 h. The resulting supernatant (cytosolic fraction) as well as the pellet (resuspended in lysis buffer B and designated as the microsomal fraction), was used for protein detection of calpain.

2.7. Immunodetection

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of cellular proteins was carried out according to Laemmli [10%: PERK, p-PERK, IRE1α, p-IRE1α, Bip, calpain and calpastatin; 15%: eIF2α, p-eIF2α, ATF6, CHOP, caspase-4 and -12] [24]. Proteins (40 μg) were quantified according to Bradford [25] and then solubilized in Laemmli sample buffer. Electrophoresis was carried out at a constant voltage of 125 V. Cellular proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using a MilliBlot Electrobolter 1 apparatus (Milli-pore, Bedford, MA, USA) [22]. The membranes were probed with primary antibodies (see above proteins) and anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA), in Tris-buffered saline (50 mM Tris base, 150 mM NaCl, 0.1% Tween-20) (TBS-T) containing 1% bovine serum albumin (BSA) for 1 h at room temperature. Membranes were washed and incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated anti-mouse, anti-rabbit, or anti-goat IgG (Biosource, Camarillo, CA, USA) diluted in TBS-T containing 5% milk powder. Proteins were detected using the ECL plus chemiluminescence kit (PerkinElmer, Boston, MA, USA). Protein expression was analyzed using a scanning densitometer (Molecular Dynamics, Sunnyvale, CA, USA), relative to GAPDH.

2.8. Determination of caspase or calpain activity by fluorescence spectroscopy

Cells were pretreated with or without calcium chelator BAPTA-AM, or inhibitors of calpain, caspase-7, caspase-4 and caspase-12, and then exposed to H_2O_2. After the incubation, cells were washed, resuspended and then lysed at −80 °C for 30 min. The kinetic reaction was followed for 30 min after addition of the appropriate caspase or calpain substrates at 37 °C using a Quadruple Monochromator Microplate Reader (Infinite M1000, Tecan US, NC, USA). Caspase or calpain activities were measured by cleavage of the following fluorogenic peptide substrates: Suc-LEY-AMC for calpain, Ac-DEVD-AMC for caspase-3, Ac-LEVD-AFC for caspase-4, MCA-Val-Asp-Gln-Val-Gly-Lys(Lys-(DNP)-NH2 for caspase-7, Ac-ATAD-AFC for caspase-12 and Ac-LEHD-AFC for caspase-9 (Calbiochem) [22]. Activities of caspases or calpain were represented as relative cumulative fluorescence of the kinetic reaction and compared to untreated controls.

2.9. Small interfering RNAs (siRNAs)

HeLa cells (0.25×10^6) were seeded in 6-well plates and allowed to reach 60–80% confluence on the day of transfection. The small interfering RNA (siRNA) constructs, PERK siRNA (sc-36213), the non-targeting siRNA control (sc-37007) and control siRNA-FITC conjugate A (sc-36868) were obtained from Santa Cruz. Cells were transfected for 48 h with 50 pmol of each siRNA construct, according to the manufacturer’s protocol. Transfection efficiency (80%) was determined by counting FITC-positive cells by flow cytometry. Protein expression was determined in whole cell lysates (Section 2.5) of transfected cells by 10% SDS-PAGE and Western blotting (Section 2.7) using anti-PERK antibody (1:1000) (Santa Cruz). Transfected cells were made thermotolerant or non-thermotolerant and then analyzed for H_2O_2-induced apoptosis.

2.10. Statistics

Data represent means±SEM from at least 3 independent experiments performed in duplicate. When not shown, error bars lie within symbols. Comparisons among multiple groups were made by one-way ANOVA, which measures the linear contrast of means. The Bonferroni–Holm’s adjustment was used to control for the Family-wise error rate at a desired level (α=5%). Software used was JMP Statistical Discovery 4.0 (SAS Institute Inc., Cary, NC). For significant differences, P<0.05.

3. Results

3.1. Mild thermotolerance developed at 40 °C protects HeLa cells against H_2O_2-induced apoptosis and clonogenic cell killing

Morphological analysis of nuclear chromatin condensation shows that exposure of cells to H_2O_2 (25 and 50 μM) for 3 h induced cell death by apoptosis (Fig. 1Ab–Ac, B), compared to the untreated control (Fig. 1A, B). Very few (<5%) necrotic cells were seen under these conditions. The treatment of HeLa cells at a mild temperature of 40 °C for 3 h caused thermotolerance, which afforded significant protection against H_2O_2-induced apoptosis (Fig. 1Ac–Af, B). Thermotolerant cells were also resistant to H_2O_2-induced clonogenic cell killing (Fig. 1C).

3.2. Mild thermotolerance (40 °C) induces the UPR in HeLa cells

Mild thermotolerance developed at 40 °C in HeLa cells induced several Hsps and antioxidants [13,19]. These defense proteins could be involved in the protective effect of thermotolerance against H_2O_2-induced apoptosis [19]. In addition, the ability of mild thermotolerance (40 °C) to evade H_2O_2-induced apoptosis might be associated with the activation/up-regulation of other survival responses such as ER stress. This study therefore determines whether mild thermotolerance (40 °C) can induce the expression and/or activation of adaptive proteins of the UPR such as PERK, IRE1α and ATF6. Activation of PERK by oligomerization and autophosphorylation to its active form p-PERK, in turn phosphorylates eukaryotic initiation factor 2 (eIF2α), attenuating the translational process to reduce the load of newly synthesized proteins in the ER lumen [26]. Indeed, the exposure of HeLa cells to mild heat (40 °C) for 3 h caused significant activation (phosphorylation) of PERK (Fig. 2A, B) and eIF2α (Fig. 3A, B), compared to non-thermotolerant control cells. The levels of p-PERK and eIF2α were increased by 2.4- and 1.5-fold, respectively. In addition, protein expression of PERK (Fig. 2C, D) and eIF2α (Fig. 3C, D) increased by 1.4- and 1.2-fold, respectively. There was a small 1.2-fold increase in protein expression of the ER chaperone Bip, compared to the non-thermotolerant control (Fig. 4A, B). The levels of p-IRE1α, p-eIF2α (Fig. 7C), ATF6 and cleaved ATF6 (Fig. 8A, B) were unchanged in mild thermotolerant cells. Together, these results show that mild thermotolerance enhanced the pro-survival effects of the PERK/eIF2α branch of the UPR.

3.3. Activation of the UPR by H_2O_2 in HeLa cells: role of mild thermotolerance

The exposure to milder conditions of stress can often induce cellular defenses to enable the cell to survive. Therefore, the ability of H_2O_2...
to activate the UPR, under mild conditions, was evaluated in non-thermotolerant HeLa cells. The activation status of the ER stress sensors PERK, ATF6 and IRE1α was evaluated, as well as expression of chaperone Bip (Figs. 5–9). The treatment of cells with H2O2 (15–50 μM) for 15 min caused significant and dose-dependent activation (phosphorylation) of PERK (Fig. 5A, B), eIF2α (Fig. 6A, B) and IRE1α (Fig. 7A, B), relative to untreated controls. H2O2 caused cleavage of ATF6, which is shown by a decrease in protein expression of its 90 kDa native form (Fig. 8A, B) and an increase in its 36 kDa cleavage fragment (Fig. 8C, E), relative to untreated controls. H2O2 induced protein expression of Bip after 15 min (Fig. 9A, C). These early ER stress responses were essentially lost after a longer 30 min treatment with H2O2 (data not shown). Together, these results show that exposure to mild stress induced by H2O2 activates the 3 branches of the UPR in HeLa cells.

Subsequently, the ability of mild thermotolerance (40 °C, 3 h) to modulate activation of the UPR by H2O2 was determined (Figs. 5–9). As mentioned above (Section 3.2), non-phosphorylated and phosphorylated forms of PERK (Fig. 2) and eIF2α (Fig. 3) were higher in thermotolerant versus non-thermotolerant cells. However, when thermotolerant cells were treated with H2O2, there were no significant

Fig. 1. Mild heat pretreatment at 40 °C causes resistance to H2O2-induced apoptosis and clonogenic cell killing. (A) (a to c) Normal (non-thermotolerant) (37 °C, 3 h) and (d to f) thermotolerant (40 °C, 3 h) HeLa cells (10^6/ml) were incubated with H2O2 concentrations of 0 μM (a, d), 25 μM (b, e), and 50 μM (c, f) for 3 h. (B) The fractions of apoptotic (Hoechst 33258) cells are given relative to the total number of cells (magnification 320×). Necrotic cells, stained with PI, were <5%. (C) Clonogenic cell survival is shown for H2O2-treated thermotolerant versus normal cells. The control value represents 10^5 cells and was normalized to represent 100% cell survival. Data represent means and SEM from three independent experiments performed with multiple estimations per point. **P < 0.01 or ###P < 0.001 indicates a statistically significant difference between treatments (±thermotolerance) for a given concentration of H2O2.

Fig. 2. Induction and activation of PERK in cells at a mild temperature of 40 °C. Western blot analysis of (A) p-PERK and (C) PERK in whole cell lysates of non-thermotolerant (37 °C, 3 h) and thermotolerant (40 °C, 3 h) HeLa cells. Representative blots are from four individual experiments. Densitometric analyses for expression of (B) p-PERK and (D) PERK are relative to untreated controls, designated as 100%, using GAPDH as loading control. Data represent means and SEM from four independent experiments performed with multiple estimations per point. **P < 0.01: statistically significant difference compared to the untreated control.

Fig. 3. Mild thermal stress (40 °C) causes induction and phosphorylation of eIF2α. Immunodetection of (A) p-eIF2α and (C) eIF2α in whole cell lysates of non-thermotolerant and thermotolerant cells, using GAPDH as loading control. Representative blots are from three individual experiments. Densitometric analyses for expression of (B) p-eIF2α and (D) eIF2α (±SEM) are relative to untreated controls (100%). *P < 0.05: statistically significant difference compared to the untreated control.
changes in levels of p-PERK (Fig. 5B, C), PERK (Fig. 5C, D), p-eIF2α (Fig. 6B, C) and eIF2α (Fig. 6C, D), compared to untreated non-thermotolerant controls. In general, levels of these proteins were higher in H2O2-treated thermotolerant cells compared to their non-thermotolerant counterparts (Figs. 5B, D and 6B, D), except for PERK expression at 50 μM H2O2. For IRE1α, H2O2-induced increases in expression of p-IRE1α and IRE1α (Fig. 7A) were inhibited in thermotolerant cells (Fig. 7B, C, D). For ATF6, there was a dose-dependent increase in expression of the 90 kDa form when thermotolerant cells were treated with 15–50 μM H2O2 (Fig. 8A, B). Levels of ATF6 were significantly higher in H2O2-treated thermotolerant cells, compared to non-thermotolerant cells. The generation of the cleaved form of
ATF6 (36 kDa) by H2O2 (Fig. 8C) was partially decreased in thermotolerant cells (Fig. 8D, E). Levels of Bip increased slightly in H2O2-treated thermotolerant cells (Fig. 9B, C). Together, these results show that the PERK/eIF2α branch of the UPR remains activated in H2O2-treated thermotolerant cells. In addition, protein levels of ATF6 (90 kDa) were

**Fig. 8.** H2O2 causes cleavage of ATF6: role of mild thermotolerance. (A, C) Non-thermotolerant (37 °C, 3 h) and (A, D) thermotolerant (40 °C, 3 h) cells were incubated for 15 min at 37 °C with H2O2 (0–50 μM) in D-MEM containing 10% FBS. Representative Western blots for (A) ATF6 (90 kDa) and (C, D) cleaved ATF6 (36 kDa) are from three independent experiments, using GAPDH as loading control. Densitometric analyses for expression of (B) ATF6 (90 kDa) and (E) cleaved ATF6 (36 kDa) (±SEM) are relative to the untreated non-thermotolerant controls at 37 °C (100%). #P<0.05 or ##P<0.01: statistically significant difference between treatments (±thermotolerance) for a given concentration of H2O2.

**Fig. 9.** Effect of H2O2 on expression of Bip. HeLa cells were incubated with H2O2 (0–50 μM) for 15 min. Representative Western blots for Bip (78 kDa) in (A) non-thermotolerant and (B) thermotolerant cells (+normal control at 37 °C) are from three individual experiments, relative to GAPDH. (C) Densitometric analysis for Bip expression (±SEM) is relative to the untreated non-thermotolerant control (100%).

ATF6 (36 kDa) by H2O2 (Fig. 8C) was partially decreased in thermotolerant cells (Fig. 8D, E). Levels of Bip increased slightly in H2O2-treated thermotolerant cells (Fig. 9B, C). Together, these results show that the PERK/eIF2α branch of the UPR remains activated in H2O2-treated thermotolerant cells. In addition, protein levels of ATF6 (90 kDa) were

**Fig. 10.** H2O2 increases CHOP expression in HeLa cells: protective role of thermotolerance (40 °C). Non-thermotolerant and thermotolerant cells were treated with H2O2 (0–50 μM) for 2 h. (A) A representative Western blot is shown for CHOP (28 kDa) expression from four independent experiments, using GAPDH as loading control. (B) Densitometric analysis for CHOP expression (±SEM) is relative to the untreated non-thermotolerant control (100%). *P<0.05: statistically significant difference between treatment with H2O2 and the untreated control. #P<0.05: statistically significant difference between treatments (±thermotolerance) for a given concentration of H2O2.
induced by H$_2$O$_2$ in thermotolerant cells, whereas cleavage of ATF6 and activation of IRE1$\alpha$ by the oxidant were decreased.

3.4. H$_2$O$_2$ induces ER-mediated apoptosis in HeLa cells

3.4.1. H$_2$O$_2$ up-regulates pro-apoptotic factor CHOP: effect of mild thermotolerance

Severe and prolonged ER stress generally results in cell death via apoptosis. The pro-apoptotic transcription factor CHOP (also known as growth-arrest and DNA-damage-inducible gene 153 (GADD 153)) is strongly induced in response to ER stress [7]. Exposure of HeLa cells to pro-oxidant H$_2$O$_2$ (25, 50 $\mu$M) for a longer time of 2 h caused a significant 1.2 to 1.6-fold increase in CHOP expression compared to untreated control cells (Fig. 10A, B). Mild thermotolerance (40 °C) completely inhibited the induction of CHOP expression by H$_2$O$_2$.

3.4.2. Activation of calpain by H$_2$O$_2$: role of mild thermotolerance

The alteration of ER calcium homeostasis can result in activation of cytosolic proteases known as calpains. They play a major role in ER-mediated apoptosis by processing and activating the ER initiators caspase-12 and caspase-4 [27,28]. We therefore determined if calpains are involved in ER-mediated apoptosis during exposure of HeLa cells to H$_2$O$_2$. Calpain activity is regulated by its endogenous inhibitor, calpastatin. Treatment of cells with H$_2$O$_2$ resulted in calpastatin cleavage (Fig. 11). Levels of the 110 kDa cleaved fragment increased by 1.45 to 1.8-fold as a function of H$_2$O$_2$ concentration, compared to untreated control cells (Fig. 11A, B). Calpastatin cleavage by H$_2$O$_2$ was significantly attenuated in mild thermotolerant cells (Fig. 11A, B). As a consequence, there was a significant increase in enzymatic activity of calpains upon treatment with H$_2$O$_2$ (Fig. 12A). Active calpains are found predominantly at the plasma membrane.
Indeed, increased protein levels of calpain were detected in microsomal fractions (Fig. 12B, C) of H$_2$O$_2$-treated cells, along with a corresponding decrease in levels in cytolsolic fractions (Fig. 12D, E). Mild thermotolerance significantly diminished H$_2$O$_2$-induced calpain activation (Fig. 12A), and its translocation to the plasma membrane (Fig. 12B–E).

3.4.3. Roles of calcium, calpain and caspase-7 in activation of ER caspases-4 and -12 during exposure to H$_2$O$_2$: protective role of mild thermotolerance

Caspase-12 (mice) and -4 (human) have been proposed as caspases that initiate ER stress-induced apoptosis [3,10]. Pro-caspase-12 is localized at the cytosolic side of the ER membrane and can be activated through several mechanisms, including cleavage by m-calpain and by caspase-7 [6,27–29]. Treatment of HeLa cells with H$_2$O$_2$ for 2 h led to a significant increase in enzymatic activities of caspase-4 (Fig. 13A) and caspase-12 (Fig. 13B). H$_2$O$_2$ caused a significant increase in levels of the active cleavage fragments of caspase-4 (20 kDa) (Fig. 13C, D) and caspase-12 (33 kDa) (Fig. 13E, F). The activation of caspases-4 and -12 was inhibited in mild thermotolerant (40 °C) cells (Fig. 13A–F).

Subsequently, the upstream factors responsible for activation of ER caspases were determined. During ER stress, caspase-7 associates with procaspase-12 and can cleave the pro-domain to initiate the processing of caspase-12 [30]. A significant increase in enzymatic activity of caspase-7 activation was seen in HeLa cells exposed to H$_2$O$_2$ for 2 h (Fig. 14A). No activity was detected after a shorter 1 h exposure to H$_2$O$_2$. Mild thermotolerance significantly attenuated H$_2$O$_2$-induced caspase-7 activation (Fig. 14A). The activation of caspase-4 (Fig. 14B) and caspase-12 (Fig. 14C) was significantly inhibited by a calcium chelator (BAPTA-AM), and by inhibitors of calpain and caspase-7. This confirms the roles of calcium, calpain and caspase-7 as activating factors of the ER caspases-4 and -12.

Once activated, caspase-12 can activate caspase-9, which in turn can catalyze cleavage of procaspase-3 [31]. The roles of caspase-12 and caspase-4 in the activation of caspase-9 and caspase-3 in H$_2$O$_2$-treated cells were evaluated by means of inhibitors of caspase-4 and caspase-12. The activation of caspase-9 was decreased partially, but not completely, by inhibitors of caspase-4 and caspase-12 (Fig. 15A). These results suggest that caspase-12 and -4 can directly activate caspase-9, without the involvement of cytochrome c and mitochondria, during H$_2$O$_2$-induced apoptosis. In a similar manner, the inhibitors of caspase-4 and -12 partially inhibited the activation of executioner caspase-3 by H$_2$O$_2$ (Fig. 15B). Furthermore, BAPTA-AM and the calpain inhibitor also caused partial inhibition of caspase-3 activation by H$_2$O$_2$ (Fig. 15B). H$_2$O$_2$-induced apoptosis (chromatin condensation) was also partially decreased by BAPTA-AM, and inhibitors of calpain and caspases-7, -4 and -12 (Fig. 15C). These findings suggest that the activation of caspase-3 and the induction of apoptosis by H$_2$O$_2$ are dependent, in part, on the disruption of calcium homeostasis and apoptosis mediated by the ER.

3.4.4. Role of the ER stress response in resistance to H$_2$O$_2$-induced apoptosis

We subsequently determined if a causal connection exists between the ER stress response and resistance to H$_2$O$_2$-induced apoptosis. Therefore, levels of PERK were decreased to evaluate whether the resistance to H$_2$O$_2$-induced apoptosis seen following exposure to mild hyperthermia could be inhibited. Transfection of cells with a siRNA targeted against PERK led to a decrease in protein levels of PERK to 60% of control levels (Fig. 16A, B). Cells with control or decreased levels of PERK were then made thermotolerant or non-thermotolerant, and exposed to 50 μM H$_2$O$_2$ for 3 h. Knock down of PERK had little effect on H$_2$O$_2$-induced apoptosis in non-thermotolerant cells. However, in thermotolerant cells with decreased levels of PERK, there was partial reversal of...
resistance to H$_2$O$_2$-induced apoptosis, compared to scrambled siRNA controls (Fig. 16C, D). This shows that the ER stress response plays a partial role in resistance to H$_2$O$_2$-induced apoptosis following mild heat preconditioning.

4. Discussion

4.1. Induction of the UPR by mild thermotolerance (40 °C) in HeLa cells

This study shows that mild thermotolerance developed at 40 °C can elicit the ER stress response. Mild heat stress enhanced the pro-survival effects of the PERK/elf2α branch of the UPR. Adaptive responses induced by pre-exposure of cells and tissues to low levels of stresses often induce cellular defenses including stress proteins such as Hsps, antioxidants (SOD, catalase), anti-apoptosis proteins (e.g. Bcl-2, inhibitor of apoptosis proteins (IAPs), cellular FLICE-inhibitory protein (cFLIP)) and damage repair molecules, as well as survival signaling pathways involving extracellular regulated kinases 1/2 (ERK1/2) and phosphatidylinositol 3-kinase (PI3-K)/Akt [3,9,32]. In HeLa cells, mild heat stress (40 °C, 3 h) triggered the induction of Hsps [20] and antioxidants (SOD, catalase, glutathione) [19]. This study shows further that mild thermotolerance (40 °C) can activate another cell survival mechanism, the PERK/eIF2α branch of the UPR. The phosphorylation of eIF2α allows translation of ATF4, which induces the transcription of survival genes involved in amino acid metabolism, redox reactions, the stress response, and protein secretion [33]. Once activated, PERK can mediate an anti-apoptotic response by phosphorylating nuclear factor (erythroid-derived 2)-like 2 (Nrf2), a transcription factor that promotes cell survival in response to ER stress [34]. The phosphorylation of Nrf2 by PERK activates the transcription of protective genes containing antioxidant responsive elements (AREs) such as NAD(P)H:quinone oxidoreductase 1, heme oxygenase 1, glutathione S-transferase and the rate limiting enzyme in glutathione biosynthesis, γ-glutamylcysteine synthetase (γ-GCS) [35–38]. Interestingly, mild thermotolerance increased protein expression of γ-GCS in HeLa cells [19], which could be a consequence of PERK activation.

4.2. Lower doses of H$_2$O$_2$ activate the UPR while higher doses cause apoptosis through the ER

ROS cause protein damage, which could lead to accumulation of misfolded proteins in the ER lumen. Mild oxidative stress (H$_2$O$_2$) (15–50 μM, 15 min) activated the three ER stress sensors, PERK, IRE1α and ATF6 in HeLa cells. The activation of these sensors increases the protein folding capacity of the cell by promoting gene transcription for ER chaperones and ER-associated degradation of damaged proteins [7,8]. H$_2$O$_2$ (50 to 500 μM, 24 h) caused ER stress (induction of GRP78/Bip, p-PERK and p-eIF2α) in human oral keratinocytes and oral cancer cells [39]. In mesenchymal stem cells, H$_2$O$_2$ (120 μM, 6 to 24 h) caused an increase in the expression of Bip [40]. This study shows that the ER stress survival response predominates under milder exposure conditions (15 min) to H$_2$O$_2$ (15–50 μM), whereas apoptosis predominates during more severe conditions.

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Fig. 14. Roles of calcium, calpain and caspase-7 in H$_2$O$_2$-induced activation of caspase-4 and caspase-12. Non-thermotolerant (37 °C, 3 h) and thermotolerant (40 °C, 3 h) HeLa cells (1×10$^6$/ml) were exposed to H$_2$O$_2$ (0–50 μM) for 1 or 2 h at 37 °C. (A) Caspase-7 activity in cell lysates is relative to the untreated non-thermotolerant control at 37 °C (100%). Non-thermotolerant (37 °C, 3 h) and thermotolerant (40 °C, 3 h) cells, with or without pretreatment (1 h) with inhibitors of calpain (20 μM), caspase-7 (50 μM), or a calcium chelator (BAPTA-AM, 50 μM), were exposed to H$_2$O$_2$ (0–50 μM) for 2 h. Enzymatic activities of (B) caspase-4 and (C) caspase-12 are given relative to untreated non-thermotolerant controls at 37 °C (100%). Data represent means and SEM from three independent experiments performed with multiple estimations per point. **P<0.01: statistically significant difference between treatment with H$_2$O$_2$ and the untreated control.#P<0.05, ##P<0.01 or ###P<0.001: statistical difference between treatments (A) (±thermotolerance) or (B, C) (±inhibitor) for a given concentration of H$_2$O$_2$. 

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oxidative stress (1–3 h). ER-mediated apoptosis involved increased expression of CHOP, cleavage of calpastatin, and activation of calpain, caspase-4 and -12. Caspase-4 cleavage and up-regulation of CHOP were induced by peroxide (200 to 500 μM, 24 h) in human oral keratinocytes and oral cancer cells [39]. H2O2 (120 μM, 6 to 24 h) caused cleavage of procaspase-12 in mesenchymal stem cells [40]. The pro-

Fig. 15. H2O2-induced activation of caspase-9, caspase-3 and apoptosis is diminished by apoptotic protease inhibitors. Normal HeLa cells (37 °C), with or without pretreatment (1 h) with inhibitors of caspase-4 (20 μM), caspase-12 (10 μM), caspase-7 (50 μM) or calpain (20 μM), or a calcium chelator (BAPTA-AM, 50 μM), were exposed to H2O2 (0–50 μM) for 2 h (caspases) or 3 h (apoptosis) at 37 °C. Enzymatic activities of (A) caspase-9 and (B) caspase-3 are given relative to untreated non-thermotolerant controls at 37 °C (100%). (C) The fractions of apoptotic (Hoechst 33258) cells are given relative to the total number of cells. Data represent means and SEM from three independent experiments. ***P<0.001 or ****P<0.001 indicates a statistically significant difference between treatment with H2O2 and the untreated control.

Fig. 16. Knock down of PERK by siRNA causes partial reversal of resistance to H2O2-induced apoptosis following mild heat preconditioning. Cells were transfected with a siRNA scrambled control (Ctrl scr) and a siRNA directed against PERK (Santa Cruz) for 48 h. Non-transfected control cells are also shown. (A) Protein expression of PERK was evaluated by Western blotting and (B) analyzed by densitometry, relative to GAPDH. ***P<0.001. (C, D) Cells were transfected with siRNA PERK or siRNA scrambled control for 48 h and then rendered thermotolerant (40 °C, 3 h) or non-thermotolerant (37 °C, 3 h). Cells were treated with or without H2O2 (50 μM) for 3 h. (C) Apoptosis was detected by Hoechst staining and (D) the fraction of apoptotic cells is given. ###P<0.001 for scrambled siRNA control versus siRNA PERK at 40 °C for 50 μM H2O2. &&&P<0.001 represents a statistically significant difference between treatments (±thermotolerance) for 50 μM H2O2.
oxidant tert-butylhydroperoxide (tBOOH) caused calpain-mediated cleavage of procaspase-12 at the ER and its translocation to the nucleus, where increased caspase-12 activity was found, in rat hepatocytes [41].

The exact mechanisms that regulate apoptosis through the ER are not well understood. Several different pathways have been implicated including the caspase-12/caspase-4 pathway, CHOP, and IRE1-JNK pathways [3]. Although caspases-12 and -4 have been implicated in ER stress-induced apoptosis, the events responsible for their activation remain ill-defined. This study shows that calcium, calpain and caspase-7 are among the initiating factors for the caspase-12/caspase-4 pathway in H_2O_2-induced apoptosis through the ER in HeLa cells.

The role of caspase-12 in ER-mediated apoptosis is well established in mice [42]. However, its role in apoptosis of human cells is unresolved, since the human caspase-12 gene contains several inactivating mutations [43]. Despite this, caspase-12 activation has been detected in several human cell lines, including retinal pigment epithelial (ARPE-19) [44], adenocarcinomic alveolar basal epithelial (A549) [45], bronchial epithelial [46], embryonic kidney (HEK293T) [47] and HeLa cells [48,49]. Our results are in concordance with these studies. Once activated, caspase-12 causes cytochrome c-independent caspase-9 activation, followed by caspase-3 activation [31,50]. The partial attenuation of caspase-9 activation by a caspase-12 inhibitor suggests that caspase-9 can be activated by alternative pathways in H_2O_2-treated HeLa cells. Indeed, H_2O_2 caused caspase-9 activation through release of cytochrome c from mitochondria in HeLa cells [19]. In mesenchymal stem cells, H_2O_2 induced apoptosis through both mitochondrial and ER pathways [40].

Caspase-4 is considered to fulfill the function of caspase-12 in human cells [51,52]. The activation of caspase-4 was mediated by calcium and calpain during ER stress caused by tunicamycin and thapsigargin in SK-N-SH cells [53]. However, little information is available about putative caspase-4 substrates. It was reported that caspase-4 can cleave actin, ataxin and the 65 kDa subunit of splicing factor U2AF [54]. The inhibition of caspase-9 activation by a caspase-4 inhibitor in our study suggests that caspase-4 cleaved caspase-9 in a cytochrome c-independent manner, similar to caspase-12. The over-expression of caspase-4 in COS-7 cells resulted in caspase-9 cleavage in the absence of cytochrome c release from mitochondria [55].

The transcription of CHOP can be induced by the three ER sensors, but the PERK/eIF2α branch of the UPR is required for up-regulation of CHOP protein expression [7,33]. However, the details of the apoptosis pathway downstream of CHOP are not well understood. CHOP appears to be linked to apoptosis by down-regulating Bcl-2 expression [56] and inducing the expression of BH3-only protein Bim [57].

### 4.3. Mild thermotolerance as an adaptive apoptosis-resistant phenotype

This study shows that mild thermotolerance developed at 40 °C inhibited all of the pro-apoptotic events induced by H_2O_2 at the level of the ER, as well as clonogenic cell killing. Furthermore, the PERK/eIF2α branch of the UPR contributes in part to the apoptosis-resistant phenotype in HeLa cells. However, other mechanisms must also contribute to the apoptosis-resistant phenotype and these could involve increased levels of Hsps and antioxidants [19,20]. Mild hyperthermia induces catalase and peroxidase pathways that metabolize H_2O_2 [19]. These pathways could account for the resistance to H_2O_2 following mild hyperthermia, independent of the ER stress response. The ability of Hsps to inhibit apoptosis through the mitochondrial and death receptor pathways has been well characterized [58]. Hsp72 was shown to bind to IRE1α, allowing PC-12 cells to adapt to ER stress by enhancing the pro-survival effects of the IRE1α/XBP1 branch of the UPR [59]. Hsp27 and Hsp70 can both maintain cellular redox homeostasis. Hsp27 is able to increase glutathione levels [60], reduce cytosolic ROS levels [61], increase glucose 6-phosphate dehydrogenase activity [62] and/or decrease intracellular iron levels [60].

### 4.4. Conclusion

This study improves understanding about the activation of ER stress and ER-mediated apoptosis by oxidative stress. Moreover, the adaptive response (UPR) dominates during milder oxidative stress, whereas ER-mediated apoptosis occurs during more severe stress. Furthermore, it advances knowledge about the protective effect of adaptive responses induced by mild stresses, such as fever temperatures, against the induction of apoptosis by oxidative stress through the ER.

### Abbreviations

- Ac-DEVD-AMC
- Ac-Asp-Glu-Val-Asp-AMC
- Ac-LEVD-AFC
- Ac-Leu-Glu-Val-Asp-AFC
- AFC: amino trifluorocoumarin
- AMC: amino methylcoumarin
- AP-1: activator protein-1
- ARE: antioxidant response element
- ASK1: apoptosis signaling kinase-1
- ATF6: activating transcription factor-6
- BAPTA-AM: 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid
- Bcl-2: B-cell lymphoma-2
- BSA: bovine serum albumin
- DTT: dithiothreitol
- ECL: electrochemiluminescence
- EDTA: ethylenediaminetetraacetic acid
- EGTA: ethylene glycol (2-aminoethylether)-N,N,N′,N′-tetraacetic acid
- eIF2α: eukaryotic initiation factor 2 alpha
- ER: endoplasmic reticulum
- ERAD: ER-associated protein degradation
- ERK1/2: extracellular regulated kinases 1/2
- FBS: fetal bovine serum
- c-FliP: cellular FLICE-inhibitory protein
- FMK: fluoromethyl ketone
- GAPDH: glyceraldehyde-3-phosphate dehydrogenase
- γ-GC5c: gamma-glutamyl cysteine synthetase
- GRP: glucose related protein
- HeLa: human cervical carcinoma cells
- H_2O_2: hydrogen peroxide
- Hsps: heat shock proteins
- IAPs: inhibitor of apoptotic proteins
- IRE1: inositol-requiring protein-1
- JNK: c-Jun N-terminal kinase
- MnSOD: manganese superoxide dismutase
- MOPS: 3-(N-morpholino)-propane sulfonic acid
- NF-κB: nuclear factor-κB
- Nrf2: nuclear factor (erythroid-derived 2)-like 2
- PBS: phosphate-buffered saline
- PDI: protein disulphide isomerase
- PERK: protein kinase RNA (PKR)-like ER kinase
- PI: propidium iodide
- PI3-K: phosphatidylinositol 3-kinase
- PMSF: phenylmethylsulfonyl fluoride
- PVDF: polyvinylidene difluoride
- ROS: reactive oxygen species
- SOD: superoxide dismutase
- UPR: unfolded protein response
- Z-ATAD-FMK: benzyloxycarbonyl-Ala-Thr-Asp-FMK
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