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Susceptibility of epithelial tumour cell lines to hyperthermia

Background: Human skin or mucosa exposes cells to both an internal and exogeneous thermal environment and the cells survive within a certain range of temperature. Exogeneous hyperthermia has been applied for the treatment of various types of cancers, fungal disease, and warts. *Objectives*: To determine whether different cellular components in the skin adapt to hyperthermic conditions differentially and further elucidate the mechanisms involved. *Materials & methods:* Cell lines derived from normal and tumour epithelial cells were treated with hyperthermic conditions and tested for viability (using an MTS assay), apoptosis (using a FITC-conjugated annexin V apoptosis detection kit), and changes in intracellular calcium (using a calcium-sensitive fluorescent single-wavelength dye, Fluo-4 AM). *Results:* Thermo-resistance of different cell types was different when cells were subjected to heat at 45°C for 30 minutes. Stronger effects of hyperthermia were noted on cell viability and apoptosis in epidermal cells relative to their malignant counterparts, except for cell lines harbouring human papillomavirus (HPV). Hyperthermia had a much greater effect on cell viability and apoptosis in a HPV-negative cell line compared to HPV-positive cell lines. We further found that hyperthermia treatment resulted in a strong calcium influx which led to apoptotic cells. However, no obvious increase in apoptosis was observed in cells treated with the CRAC channel selective inhibitor, BTP2, before application of hyperthermia in all cell types, except three cervical cell lines harbouring HPV. *Conclusion:* We propose that hyperthermia results in a CRAC-related strong calcium influx which induces apoptosis, with the exception of HPV-positive cells.

Key words: hyperthermia, CRAC channel, calcium influx, apoptosis, HPV

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Human skin or mucosa exposes cells to both an internal and exogeneous thermal environment and the cells survive within a certain temperature range. "Hyperthermia" refers to a treatment procedure or pathological phenomenon in which the temperature of a specific body part or the whole organism is increased by a few degrees above physiological temperature [1]. With modulations of therapeutic temperature and treatment time, this technique has proven effective for the treatment of various types of cancers [2-5], fungal disease [6], and viral warts [7, 8]. Hyperthermia can selectively eliminate cancer cells by facilitating cell cycle arrest, apoptosis, necrosis, and autophagy [9], however, the underlying molecular mechanisms remain to be studied, and whether different cellular components in the skin adapt to hyperthermic conditions differentially is worthy of investigation.

Calcium influx is required for the activation of cellular function. Store-operated calcium entry (SOCE) mediates calcium influx through a calcium release-activated calcium (CRAC) channel. The CRAC is a principal calcium entry mechanism in non-excitable cells. Composed of Orai molecules in the plasma membrane, CRAC channels are activated by stromal interaction molecules (STIM) that are located in the membrane of the endoplasmic reticulum (ER). STIM proteins can sense a change in calcium concentration in the ER and activate Orai molecules. There are two subtypes of STIM proteins, STIM1 and STIM2, of which STIM1 is crucial [10].

STIM1 is an ER calcium sensor that senses levels of depleted ER calcium. In the resting state, STIM1 is uniformly distributed in the ER membrane and the calcium concentration of cytosol is at a low level (~100 nM). A decrease in calcium concentration in the ER induces STIM1 multimerization and translocation into puncta close to the plasma membrane where STIM1 multimers bind to and activate the Orai channel, leading to a vast calcium influx. During the process of immunity, calcium regulates cytoskeleton remodelling, the release of vesicle contents, and transcriptional changes. In addition, increased sustained cytosolic calcium results in cellular activation or apoptosis [11]. In recent years, it has been reported that STIM1 can also sense mild increases in body temperature independently of store depletion of calcium. The increase in temperature triggers translocation of STIM1 towards plasma membrane junctions, similar to the calcium store depletion, however, the activation of Orai channels is inhibited at elevated temperatures above 40°C. A return to a lower temperature after exposure to a higher temperature

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Table 1. General information of the cell lines used in this study.

Name	Source	Organism	Cell type	Tissue	Culture Medium*
HeLa	American type culture collection	Human	Epithelial	Adenocarcinoma	Complete growth medium: Roswell Park Memorial Institute medium (RPMI, Life technologies) supplemented with 10% foetal bovine serum (FBS, Gibco)
Caski				Epidermoid carcinoma	
SiHa					
C33A					
HaCaT	Cell Bank of the Chinese Academy of Sciences (Beijing, China)		Keratinocyte	Epidermis	
A375			Melanoma	Malignant melanoma	DMEM/F12 (Life technologies) supplemented with 10% foetal bovine serum (FBS, Gibco)
A2813					
HTB140					
PIG1			Melanocyte	Melanocyte	Medium 254, supplemented with Human Melanocyte Growth Supplementary 2 (HMGS-2) and 5% FBS

**All cells were incubated at 37°C in a humidified incubator with an atmosphere of 5% CO_2.*

leads to the activation of Orai channels which results in profound calcium influx. This "heat-off" calcium influx occurs independently of calcium store depletion. Therefore, this mechanism could be of benefit during or after fever, regulating the release of vesicle contents, cellular activation, or apoptosis [12, 13].

STIM1 is often overexpressed in human cancers such as melanoma tissues, multiple melanoma cell lines, and cervical cancer, and STIM1 overexpression typically induces a strong calcium influx [14, 15]. Physiological processes such as the regulation of reactive oxygen species (ROS), mitochondrial damage, and calcium overload can influence apoptosis [16]. Calcium overload may damage mitochondria and result in the activation of the apoptotic signalling cascade [17]. Therefore, we hypothesize that treating cells with a high temperature may lead to calcium overload via CRAC channels and a higher level of apoptosis.

In our study, we show that the thermo-resistance of diverse cell types is different when cells are subjected to a hyperthermic condition of 45°C. Hyperthermic treatment results in a strong calcium influx which leads to apoptotic cells. However, no obvious increase in apoptotic cells was observed in cells treated with the CRAC channel selective inhibitor, BTP2, before application of hyperthermia in all cell types except three HPV-positive cervical cell lines. BTP2 reduced calcium influx evoked by hyperthermia in these three cervical cell lines which harbour HPV.

Materials and methods

Cell culture

The general information of the cell lines used in this study is presented in *table 1*.

Drugs

YM-58483 (N-[4-[3,5-bis(trifluoromethyl)-1H-pyrazol-1-yl] phenyl-4-methyl-1,2,3-thiadiazole-5-carboxamide, BTP2, Selleckchen) was used as a selective CRAC channel inhibitor. BTP2 was dissolved in dimethyl sulfoxide (DMSO, Japan). The cells were treated with medium containing 3 μM or 10 μM BTP2 in DMSO (1 μL DMSO/mL medium) or medium containing DMSO (1 μL DMSO/mL medium), respectively, for different groups of cells.

Heat stress

Cells were seeded into six-well plates and cultured overnight before being treated with hyperthermia in a water bath. The bottom of the culture plates was submerged in the water and incubated at 39°C, 41°C, 42°C, 43°C or 45°C for 30 minutes. For the control group, cells were incubated in the water bath at 37°C for 30 minutes. After hyperthermic treatment, cells were returned to 37°C for recovery before being collected for analysis of apoptosis.

MTS assay

Cell viability was measured using the MTS assay (Promega, Madison, WI) according to the manufacturer's instructions. In brief, cells were seeded in 96-well plates at a density of 5,000 cells per well and cultured at 37°C with 5% CO_2. Cell viability was measured by detecting the absorbance at 490 nm at Day 0, 1, 2, 3 and 4. All assays were completed in octuplicate and repeated three times.

Flow cytometry

For analysis of apoptosis, cells were stained using a FITC-conjugated annexin V apoptosis detection kit (BD Biosciences, USA) or APC-conjugated annexin V (BD Biosciences, USA) in accordance with the manufacturer's instructions. In brief, the cells were trypsinized and washed with cold PBS, stained with APC-conjugated annexin V (5 μL) and PI (5 μL), or FITC-conjugated annexin V (5 μL) and PI (5 μL) for 15 minutes at room temperature in the dark, and then analysed by BD LSRFortessa (BD Bioscience) within an hour.

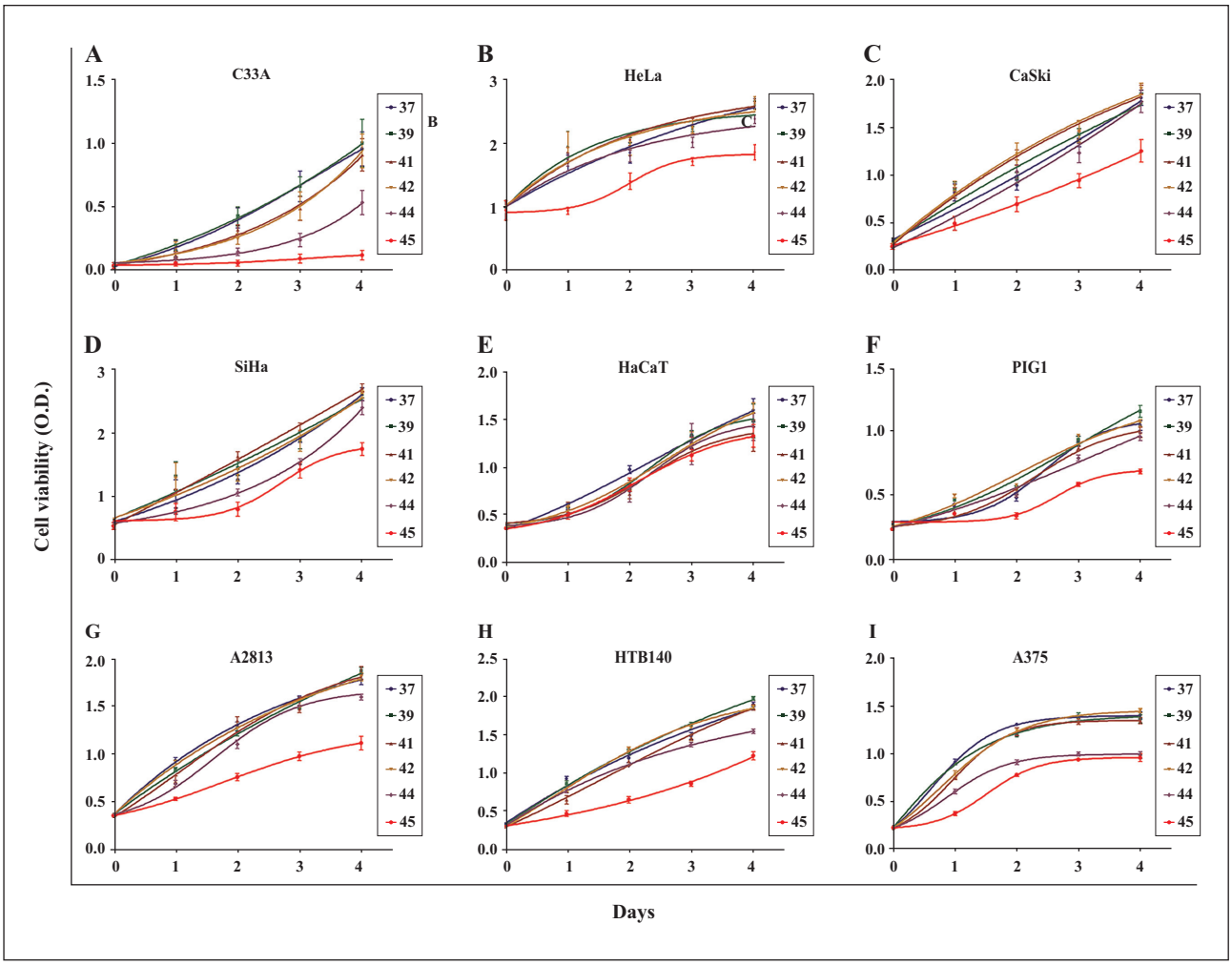

Figure 1. The effect of hyperthermia (37°C, 39°C, 41°C, 42°C, 44°C and 45°C) on cell viability, measured using an MTS assay. All cells were measured from Day 0 to Day 3 after hyperthermia. On Day 3 after hyperthermia, a change in cell viability was observed for C33A, A375, A2813 and HTB140 at temperatures above 44°C ($p<0.05$), and HeLa, Caski, SiHa, HaCaT and PIG1 above 45°C ($p<0.05$), relative to the control group.

Fluorescent calcium measurement

The calcium-sensitive fluorescent single-wavelength dye, Fluo-4 AM, was used to measure changes in intracellular calcium at 1 min, 5 mins and 120 mins post hyperthermia. In brief, cells were loaded with 5 μM Fluo-4 AM (Invitrogen) and then treated with hyperthermia for 30 mins. The fluorescent density of Fluo-4 was then measured using a fluorescence microplate reader (PerkinElmer) at 37°C. Fluorescence was evoked by 488-nm excitation wavelength and collected at 510 nm.

Statistics

The student's t test and one-way analysis of variance (ANOVA) were performed using GraphPad Prism software (Version 5.0) for statistical analysis; $p<0.05$ was considered to be statistically significant.

Results

The effect of hyperthermia on cell viability

We initially compared the thermo-sensitivity of all the cell lines to different temperatures using the MTS assay. The cell viability of untreated cells was measured on Day 0. For each cell line, the cells were divided into six groups and treated at 37°C (control group), 39°C, 41°C, 42°C, 44°C or 45°C using water baths for 30 minutes, respectively. Cell viability was measured each day from Day 1 to Day 4 after hyperthermic treatment. C33A, A375, A2813 and HTB140 showed significantly reduced cell viability at Day 4 ($p<0.05$) when treated with a temperature of 44°C (*figure 1 A, G-I*). HeLa, Caski, SiHa, HaCaT and PIG1 cell lines were most thermo-resistant and showed significantly reduced cell viability post hyperthermia at 45°C on Day 4 (*figure 1B-F*) ($p<0.05$).

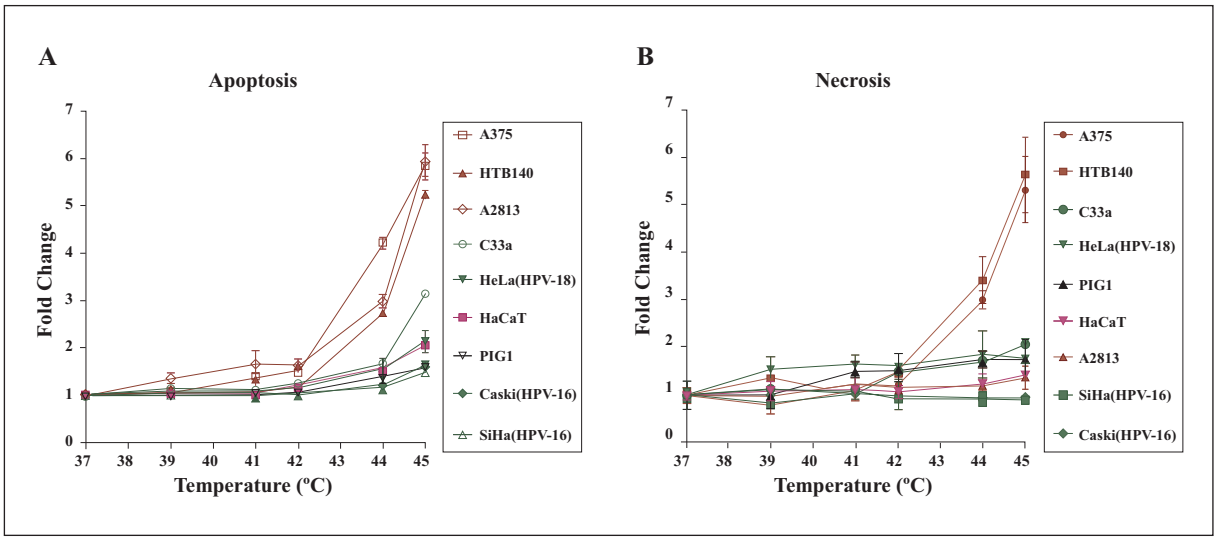

Figure 2. The effect of hyperthermia (39°C, 41°C, 42°C, 44°C and 45°C) on apoptosis and necrosis in cell lines analysed by flow cytometry.

Impact of hyperthermia on apoptosis and necrosis

Hyperthermia disrupts the integrity of the cell membrane, cytoskeleton, and mitochondrial machinery which activates apoptosis and leads to necrosis in cells [18]. In order to investigate one of the mechanisms by which hyperthermia reduces cell viability, we quantified the number of apoptotic cells by flow cytometry. Each cell line was divided into six groups and treated at temperatures of 37°C (control group), 39°C, 41°C, 42°C, 44°C and 45°C using water baths for 30 minutes, respectively. The percentage of apoptotic cells was lower for melanocyte PIG1 cells than the three melanoma cell lines after treatment with 44°C or 45°C hyperthermia ($p<0.05$). For the human HPV-negative cervical cancer cell line, C33A, significant higher percentages of apoptosis and necrosis were observed compared to the three HPV-positive human cervical cancer cell lines after treatment with hyperthermia up to 45°C ($p<0.05$). In addition, the same level of apoptosis and necrosis in each group was observed for two HPV-16-positive cervical cancer cell lines, SiHa and Caski. A higher percentage of apoptotic and necrotic cells was observed in the cervical cancer line, HeLa, which harbours HPV-18, compared to SiHa and Caski after treatment at 44°C or 45°C ($p<0.05$). A similar level of apoptosis and necrosis was observed for PIG1 and HaCaT when treated with different hyperthermic conditions (*figure 2*).

Hyperthermia-induced apoptosis in BTP2-treated cells

Using the CRAC channel selective inhibitor, BTP2, we set out to test whether hyperthermia induces cellular apoptosis via regulation of the CRAC channel. For each cell line, cells were divided into four groups: negative control group (37°C), BTP2-treated group (37°C), hyperthermia-treated group (45°C for all the cell lines), and BTP2 and hyperthermia-treated group (45°C for all the cell lines). As shown in *figure 3*, there was no difference in the number of apoptotic or necrotic cells between the negative control group (37°C) and BTP2-treated group (37°C). Hyperthermia significantly increased the apoptotic levels in most cell lines, but failed to induce intense apoptosis in BTP2 pre-treated cells, suggesting a blockade of the CRAC channel by inhibition of hyperthermia-induced apoptosis by BTP2 (*figure 3*). However, for the HPV-positive cervical cell lines, Caski, SiHa and HeLa, pre-treatment with BTP2 did not alter the percentage of apoptosis induced by hyperthermia, suggesting that hyperthermia-related calcium overload may not be the main mechanism of apoptosis in HPV-positive cervical cell lines or that rescue pathways exist in these cell lines.

Hyperthermia-induced calcium influx via CRAC activation in BTP2-treated cells

Having observed an induction of apoptosis in response to hyperthermia regardless of CRAC channel blockade in HPV-positive cell lines, we next determined whether BTP2 inhibited the CRAC channel-mediated calcium influx in response to 45°C hyperthermia in cell lines with or without HPV. As shown in *figure 4*, there was a sustained increase in intracellular calcium in C33A, Caski, HeLa and HaCaT cells, starting at one minute after hyperthermic treatment. In three cervical cancer cell lines, C33A, Caski, and HeLa, the intracellular calcium was similarly increased approximately twofold. The increase in intracellular calcium in HaCaT cells was less significant than that of the three cervical cancer cell lines, with a fold difference of 1.42, remaining at the same level at up to 120 minutes. In two melanoma cell lines, A375 and A2813, the intracellular calcium peaked at 120 minutes at about 2.2 fold. The increase in intracellular calcium in PIG1 cells was less than that in A375 and A2813 cell lines, with a fold difference of 1.87 at 120 minutes. However, based on the intracellular calcium in cell lines pre-treated with BTP2, we found that administration of 3 μM or 10 μM BTP2 significantly inhibited hyperthermia-induced increase in intracellular calcium in all cell lines in a concentration-dependent manner. These

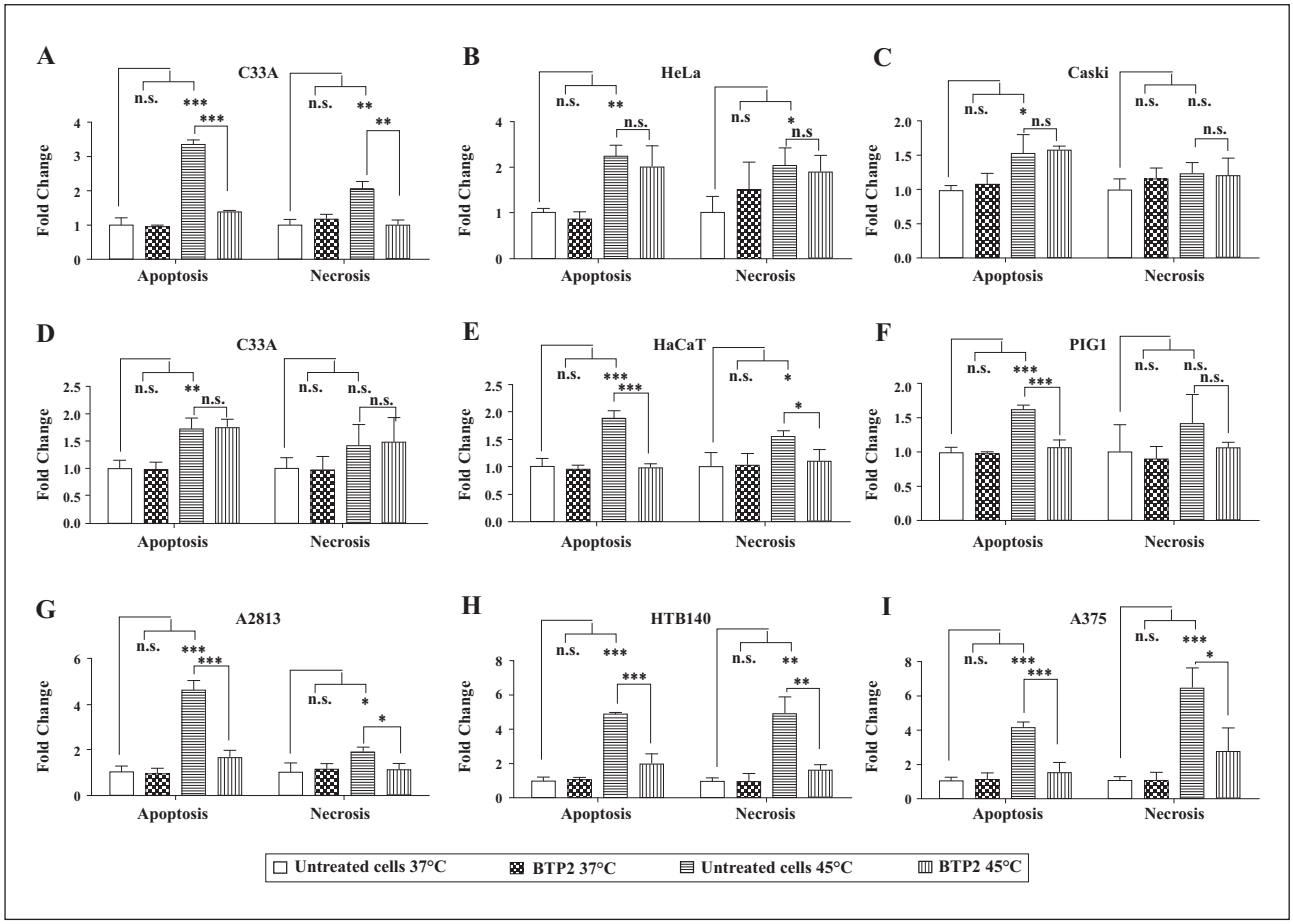

Figure 3. The effect of hyperthermia and the CRAC channel inhibitor, BTP2, on apoptosis and necrosis in cell lines, analysed by flow cytometry. Apoptotic and necrotic levels of control cells (Untreated cells 37°C) and cells subjected to 3 μM BTP2 (BTP2 37°C), hyperthermia (Untreated cells 45°C), and hyperthermia combined with 3 μM BTP2 (BTP 45°C) are shown as fold change relative to control (Untreated cells 37°C). Data shown are the means from three independent experiments ± SD (*p<0.05, **p<0.01, ***p<0.001, according to one-way ANOVA).

results suggest that hyperthermic treatment may induce calcium influx via activating CRAC channels in cells with or without HPV.

Discussion

With the advantage that heat can be delivered directly to the body surface, hyperthermia has emerged as a non-invasive therapeutic method that can target sites of diseased skin. Here, we present data suggesting that the two major cellular types of the epidermis, *i.e.* keratinocytes and melanocytes, exhibit similar thermo-resistant features when subjected to hyperthermia. Compared to "normal" cell lines, melanoma cell lines and the cervical cell line C33A showed higher levels of apoptosis after treatment with hyperthermia at 45°C. A greater increase in intracellular calcium levels was observed in C33A, HeLa and SiHa cells relative to HaCaT cells, and A375 and A2813 relative to PIG1 cells.

STIM1 and Orai1 are often abundantly overexpressed in tumour tissues and tumour cells such as human melanoma tissue, multiple melanoma cell lines, and cervical cancer. Overexpression of STIM1 usually induces a strong calcium influx [14, 15]. We conjectured that when cells were subjected to hyperthermia below 45°C, STIM1 may act as a switch converting external thermal signals to internal cellular physiological and biochemical signals by regulating calcium influx signals. Caspase-3 is the essential death protease and final executor of calcium signalling, involved in the apoptotic pathway. Survivin is a member of the inhibitor of apoptosis protein (IAP) family, and is abundantly expressed in some malignancies, but undetectable in normal adult tissues. Survivin binds to caspase-3 and inhibits caspase activity in cells exposed to diverse apoptotic stimuli [19]. Up-regulation of survivin is also associated with high-risk HPV types in both non-malignant and malignant cervical lesions [20, 21]. Survivin expression is likely to be regulated by HPV, as survivin expression level is strongly dependent on continuous HPV E6/E7 expression, and HPV E6/E7 is reported to transactivate the survivin promoter [22, 23]. Several experiments showed that survivin expression is much higher in untreated Caski, SiHa and HeLa cells than in untreated C33A cells [24, 25]. Overexpressed survivin in HPV-positive cells may account for the inhibition of caspase-3 activity, the final executor of calcium influx in apoptotic signalling, and lead to blockade of calcium channel-dependent apoptotic signalling. Therefore, alteration in calcium influx level no longer regulates blocked

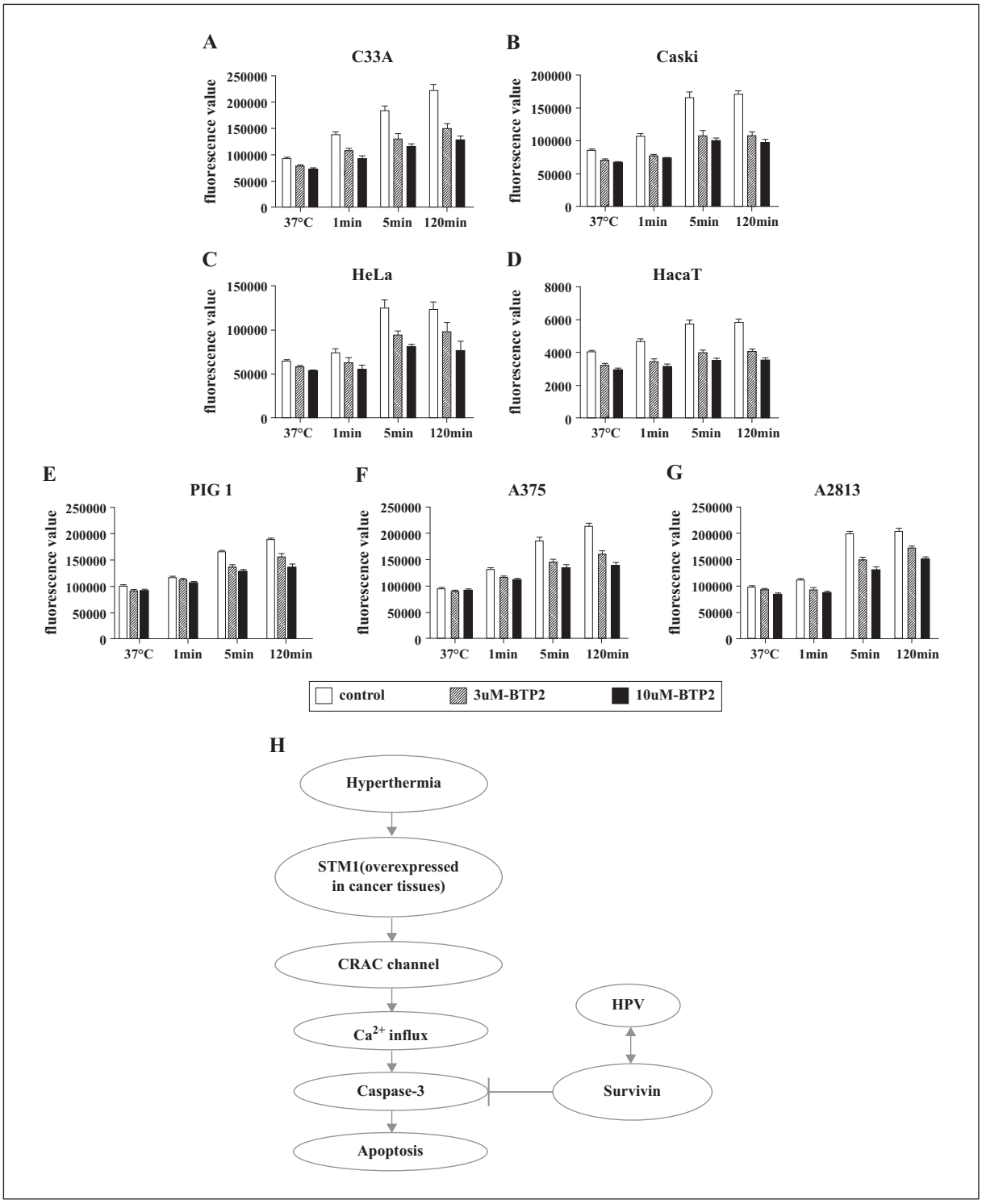

Figure 4. CRAC channel-mediated calcium influx in response to hyperthermia: **(A)** C33A cells; **(B)** Caski cells; **(C)** HeLa cells; **(D)** HaCaT cells; **(E)** PIG1 cells; **(F)** A375 cells; and **(G)** A2813 cells. Hyperthermia resulted in a strong CRAC-related calcium influx which induced apoptosis, except in HPV-positive cells. The y axis represents fluorescence values of the calcium-sensitive fluorescent single-wavelength dye, Fluo-4, used for measuring changes in intracellular calcium at a recovery time of 1 minute, 5 minutes, and 120 minutes post hyperthermia, relative to the control group (37°C). Each group was divided into three subgroups: 3 μM, 10 μM or without CRAC channel inhibitor BTP2 treatment before hyperthermia. **H)** Schematic outline of how hyperthermia results in a CRAC-related strong calcium influx which induces apoptosis. STIM1 is often abundantly overexpressed in tumour tissues, and STIM1 overexpression typically induces a strong calcium influx. Up-regulation of survivin is associated with a high-risk of HPV. Survivin binds to caspase-3 and inhibits caspase activity in cells exposed to diverse apoptotic stimuli, including hyperthermia.

calcium channel-dependent apoptotic signalling. Despite the blockade of calcium channel-dependent apoptotic signalling, apoptosis was still observed in HPV-positive cells, suggesting that other apoptotic signalling regulators may play a role. This phenomenon could explain the higher level of thermo-resistance in HPV-positive cells. Based on these findings, we hypothesize that inhibition of overexpressed survivin may further improve the therapeutic effect of hyperthermic treatment on HPV-infected lesions. The relationship between calcium influx and induction of apoptosis also suggests that the most effective temperature for treating different disease types may become apparent upon measurement of calcium influx. In future investigations, it will also be interesting to assess whether such a combination therapeutic strategy is effective for the treatment of other virus-related cancers. Our hypothesis regarding the mechanism of action of hyperthermia seems to go against its observed beneficial effects on HPV-associated warts. We conjecture that most of the HPV in cells of wart tissue exists freely within cells without chromosomal integration. However, in the cell lines we used in our experiment, HPV genes were integrated into the genome of the host. This may account for the fact that the observed results do not appear to support our hypothesis.

With a high expression level in many types of tumour cells, STIM1 is important for cancer cell proliferation, migration, and angiogenesis. STIM1 was also found to affect calpain activation and spectrin processing, and plays an important role in focal adhesion turnover. This evidence implies that overexpression of STIM1 may be a risk factor for metastasis in cancer patients [1]. Moreover, given that STIM1 overexpression may lead to high calcium influx levels in tumour cells, we hypothesize that treating STIM1-overexpressed tumour cells with hyperthermia may lead to calcium overload and high levels of apoptosis, which therefore more efficiently kills cancer cells. This finding may provide novel patient selection criteria for hyperthermia treatment. Since a higher temperature of hyperthermia causes greater calcium influx, by regulating the intensity of calcium influx, we may consequently determine the most suitable hyperthermic conditions in order to obtain the best therapeutic effects, making hyperthermia therapy more individual and accurate.

To our knowledge, this is the first study to describe induction of CRAC channel-dependent calcium influx in response to hyperthermia, leading to apoptosis. We hypothesise that when cells are subjected to hyperthermia, STIM1 may act as a switch to convert external thermal signals to internal cellular physiological and biochemical signals by regulating calcium influx signals. Different levels of calcium influx provide different effects in different types of cells. ■

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