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Transient inhibition of Calyculin A induced premature chromosome condensation by hyperthermia

J. W. J. BERGS[1*], R. TEN CATE[1], H. M. RODERMOND[1], P. A. JAARSMA[1], J. P. MEDEMA[1], F. DARROUDI[2], M. R. BUIST[3], L. J. A. STALPERS[1], J. HAVEMAN[1], C. VAN BREE[1], & N. A. P. FRANKEN[1]

[1]*Laboratory for Experimental Oncology and Radiobiology (LEXOR), Department of Radiation Oncology, Center for Experimental Molecular Medicine (CEMM), Academic Medical Center, University of Amsterdam, Amsterdam,* [2]*Department of Toxicogenetics, Leiden University Medical Center, Leiden and* [3]*Department of Gynecology, Academic Medical Center, University of Amsterdam, The Netherlands*

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Abstract

The analysis of chromosomal aberrations by premature chromosome condensation (PCC) induced by Calyculin A (Cal) is feasible in tumor biopsies from patients and has the potential to predict sensitivity to radiotherapy. As hyperthermia (HT) improves radiotherapy outcome in certain tumor sites, it was investigated whether PCC induction is still possible after temperatures reached in the clinic. Human cervical carcinoma (CaSki) and lung carcinoma (SW-1573) cells were incubated with Cal to induce PCC immediately after 1 h treatment at temperatures ranging from 41°C to 43°C and after recovery for up to 24 h after treatment with 43°C. Levels of phosphorylated Cdc2 (at the Tyr15 residue), histone H3 (at the Ser10 residue) and Cyclin B1 were investigated by immunoblotting. The amount of cells positive for phosphorylated histone H3 was determined by flow cytometry. Temperatures $\geq$42.5°C inhibited the induction of PCC by Cal, while recovery of PCC-induction was observed at >20 h after treatment in both cell lines. The phosphorylation status of Cdc2 as well as of histone H3 in cells treated with Cal directly after HT at 43°C was similar to that of cells treated with Cal alone or treated with Cal 24 h after HT at 43°C. HT alone did not affect the levels of phosphorylated Cdc2, while phosphorylation levels of histone H3 were increased as compared with control status of these two proteins. Phosphorylated and total Cyclin B1 levels were not influenced by any of the treatments. Flow cytometric analysis confirmed that HT at 43°C did not interfere with phosphorylation of histone H3. Our data indicate that HT transiently inhibits PCC induction by Cal in a temperature-dependent manner. Therefore, an interval of at least 24 h after HT should be applied before taking tumor biopsies for karyogram analysis of patients treated with temperatures above 42.5°C.

Keywords: *Hyperthermia, Calyculin A, premature chromosome condensation, biology*

Introduction

The analysis of chromosomal aberrations in tumor biopsies with the potential to predict radiation treatment outcome is feasible in colorectal [1] and cervical carcinoma [2]. Premature chromosome condensation followed by fluorescent in situ hybridization (FISH) enables the direct visualization of chromosomal aberrations by the induction of chromosome condensation in interphase cells [1–4]. PCC induction can be accomplished by fusion of cells with mitotic cells or by using chemicals, such as Calyculin A [3, 4].

Cell-cycle independent induction of PCC by Cal is thought to be caused by inhibition of type-1 (PP1) and type-2 phosphatases (PP2A) [3–7]. These phosphatases are involved in the physiological regulation of chromosome condensation during mitosis by regulating the activity of the phosphatase

Correspondence: Nicolaas AP Franken, PhD, AMC-UvA LEXOR-CEMM-Radiation Oncology, P.O. Box 22700 1100 DE Amsterdam, The Netherlands. Tel: +31 20 5668769. Fax: +31 20 6091278. E-mail: n.a.franken@amc.uva.nl

*Currently: Institute of Molecular Biology and Tumor Research, Philipps-University Marburg, Emil-Mannkopff-Str. 2, D-35032 Marburg, Germany.

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Cdc25 and the maturation/mitosis promoting factor (MPF), a complex of $p34^{cdc2}$ (Cdc2) and Cyclin B [7–11]. In the inactive form of MPF, the Cdc2 protein is phosphorylated at Tyr15 (Cdc2-P_{Tyr15}) and Thr14 residues [12]. Dephosphorylation of Cdc2-P_{Tyr15} by Cdc25 is important for entry of cells into mitosis [11] and has been associated with chromosome condensation [13, 14]. In mitotic cells, phosphorylation of the histone H3 at the Ser10 residue (H3-P_{Ser10}) is described as occurring at high levels and correlates with mitotic chromosome condensation [15, 16]. H3-P_{Ser10} is used as a marker for mitotic cells and promotes the recruitment of chromosomal condensation factors necessary for normal mitotic assembly and segregation [17]. Enhanced H3-P_{Ser10} is also associated with PCC induction after incubation of cells with Cal [18–21]. Also, cyclin B1 levels may play a role in the studied effects and hyperthermia induced chromosome condensation and increases in cellular cyclin B1 levels by hyperthermia have been reported by Mackey et al. [22].

Recently, we have shown that chromosome fragments have the potential to predict radiosensitization by hyperthermia (HT) at temperatures reached in the clinic [23]. Iliakis et al. have reported previously that HT (8–15 minutes at 45.5°C) reduces the ability of chromosomes to condense after fusion with mitotic cells and suggested that accumulation of proteins on the chromatin might prevent protein phosphorylation necessary for chromatin condensation [24]. It has already been shown that HT can affect the phosphorylation status of histone proteins, such as H2AX [25–27]. The protein accumulation in the nucleus might be due to denaturation of proteins [28], when cells are treated at temperatures at or above 42.5°C. Since clinically more relevant temperatures below 42.5°C might not result in protein denaturation [28–30], the present study investigates the temperature-dependent induction of PCC by Cal and the phosphorylation status of two key regulators of chromosome condensation in two human tumor cell lines.

Materials and methods

Cell culture

The human cervical cancer cell line CaSki was grown in Dulbecco's modified Eagle medium (DMEM) with 4500 mg/L glucose (GIBCO-BRL Life Technologies, Breda, the Netherlands) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 2 mM glutamine and kept at 37°C in a humidified incubator with a 10% CO_2/90% air atmosphere. The SW-1573 cell line was grown in Leibowitz-15 (GIBCO-BRL Life Technologies, Breda, the Netherlands) supplemented with 10% FBS and glutamine, penicillin and streptomycin. The cells were maintained at 37°C in an incubator with humidified air without additional CO_2. Both cell lines were maintained as monolayers in tissue culture flasks (Costar Europe Ltd, Badhoevedorp, the Netherlands). The cultures were passaged twice a week to ensure exponential growth.

Processing of human biopsies

After surgical resection from patients, tumour biopsies were placed in phosphate buffered saline. The tumour samples were cut in small pieces and digested for 1–4 h at 37°C in high glucose DMEM supplemented with 0.14 units/ml collagenase/dispase enzyme solution (Blendzyme I, Roche, Indianapolis, IN, USA). After the digestion, samples were dislodged by pipetting the tissue up and down and centrifuged for 5 min at 68 *g*. The supernatant was removed and the pellet was resuspended in a wash medium containing high glucose DMEM supplemented with 5% FCS, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM glutamine. The samples were centrifuged for 5 min at 68 *g*, and by removing the supernatant mainly loose cells and bacteria were removed. This washing step was repeated once. The remaining organoids were transferred to CellBIND® (Corning, Lowell, MA, USA) 6-well plates in RPMI supplemented with 10% FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, 25 μg/ml gentamycin, 1 μg/ml fungizone and 2 mM glutamine and incubated at 37°C, 5% CO_2 for 1–2 days before hyperthermia treatment.

Hyperthermia treatment

Cultures consisting of approximately 49% G1, 34% S and 17% G2/M (CaSki) or 45% G1, 46% S and 10% G2/M (SW-1573) as determined by BrdU labeling and flow cytometry were treated. Hyperthermia treatment was performed by submerging the Petri dishes in a thermostatically controlled water-bath for 1 h. The atmosphere of the water-bath was adjustable by a connection with air and CO_2 supplies. CaSki cells were heated in a 10% CO_2/90% air atmosphere with an air inflow of 2 L/min. SW-1573 cells did not receive additional CO_2.

Premature chromosome condensation

To induce premature chromosome condensation (PCC), 80 nM of Calyculin A (Santa Cruz Biotechnology, CA) was added for 1 h immediately after HT at temperatures ranging from 41°C to 43°C [3]. To test whether the effect of HT at 43°C on PCC induction was transient, cells were

incubated for 0 h to 24 h at 37°C after HT treatment before the addition of Cal. Cells were collected and treated according to standard cytogenetic procedures to obtain chromosome spreads. Briefly, cell pellets were resuspended in a hypotonic solution of 75 mM KCl, incubated for 16 min at 37°C and fixated in glacial acetic acid/methanol (1:3 v/v). Chromosome spreads were obtained by dropping the fixated cells on to pre-cleaned slides and air dried. Chromosomes were stained using 10% Giemsa (Merck, Darmstadt, Germany) solution and investigated by light microscopy (Leica, Rijswijk, the Netherlands). The percentage of PCC spreads of G1 and G2 phase cells (G1 + G2 PCC index) was determined by counting 200 nuclei per slide. For each sample, at least two slides were scored. Experiments were repeated three times.

Immunoblotting

Whole-cell lysates were prepared in Laemmli buffer. Protein concentration was determined using the BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). Samples were diluted in 3 × reduced buffer before loading on to 10–15% polyacrylamide gels. Separated proteins were transferred to polyvinylidene difluoride membranes and hybridized overnight at 4°C with antibodies recognizing Cdc2-P_{Tyr15}, P-Cyclin B1 and total H3 (rabbit polyclonals 9111, 4131 and 9715, Cell Signaling Technology, Danvers, MA, USA), H3-P_{Ser10} (rabbit polyclonal 23136, Upstate Biotechnology, Lake Placid, NY, USA), total Cdc2 and Cyclin B1 (mouse monoclonal C12720 and 610219, BD transduction laboratories, Franklin Lakes, NJ, USA) or β-Actin (mouse monoclonal AC-15, Sigma-Aldrich, Zwijndrecht, the Netherlands). Membranes were probed with secondary IgG HRP-linked antibodies anti-rabbit or anti-mouse (Cell Signaling Technology, Boston, MA), incubated with Enhanced Chemiluminescence Plus (Amersham Biosciences, Little Chalfont, UK) and visualized using a ChemiDoc XRS system with Quantity One (version 4.4) software (BioRad Laboratories, Hercules, CA, USA).

Flow cytometric analysis of phosphorylation of histone H3

Cells were harvested, fixated in 70% ethanol and incubated at −20°C for at least 24 h. For flow cytometric detection of $H3P_{Ser10}$, cells were permeabilized using 0.25% Triton X-100 and incubated on ice for 15 min. Cell pellets were then resuspended in rabbit polyclonal H3-P_{Ser10} (23136) followed by 50 μg/ml FITC-conjugated goat anti-rabbit secondary antibody (Jackson Immuno Research Laboratories, Suffolk, UK). As isotype (negative) controls, cell pellets were resuspended in rabbit α-Helicobacter Pylori primary antibody (Dako, Glostrup, Denmark). Cells were then stained using 25 μg/ml of propidium iodide (PI) containing 0.1 mg/ml RNase A. Experiments were repeated three times. To define H3-P_{Ser10} positivity, a positive control consisting of mitotic cells obtained by shake-off was used as a reference by determination of a tetraploid (4N) H3-P_{Ser10} positive population. The cut-off level was used for determination of H3-P_{Ser10} positive cells in HT and/or Cal treated cultures. In addition to H3-P_{Ser10} positivity, cell cycle distribution was determined by measurement of PI content in CaSki and SW-1573 cultures. Analysis of flow cytometry data was performed by using WinMDI version 2.9.

Statistics

Data on the induction of PCC were analyzed using SPSS 14.0 (Chicago, IL) statistical software using the non-parametric Mann-Whitney test.

Results

Transient inhibition of PCC-induction by Cal in a temperature-dependent manner

To investigate the effect of HT on PCC induction by Cal, cells were incubated with Cal immediately after temperatures in the range of 41°C to 43°C. In control cultures, the total PCC-index (S-, G1- and G2-phase PCC) was 23% ± 4% (SEM) for CaSki cells and 19% ± 5% (SEM) for SW-1573 cells. The majority (for both cell lines around 60%) of the nuclei showing PCC consisted of S-phase nuclei, recognizable by their pulverized appearance (Figure 1A). From the remaining PCC, the vast majority (±98%) were G2-phase cells with two distinct chromatids (Figure 1B), whereas ±2% were G1-phase cells with one chromatid (Figure 1C).

Only in cells treated at temperatures ≥42.5°C PCC induction was significantly decreased ($p = 0.04$ for 42.5°C and $p = 0.02$ for 43°C) compared to untreated cells (Figure 1D and E). Representative examples of nuclei after incubation of SW-1573 cells with Cal without and with prior HT at 43°C are shown in Figures 1F and G respectively. In HT-treated cells, condensation of nuclei was visible, but it did not result in chromosome spreads (Figure 1F). In cells derived from biopsies of cervical tumors from several patients after *in vitro* treatment we found 1% scorable PCCs directly after HT at 43°C while in untreated biopsy-derived cells this is between 10% and 15%. These results indicate that HT at ≥42.5°C inhibits the induction of PCC by Cal.

Whether cells were able to recover from this inhibition of PCC-induction was investigated by incubating cells with Cal at various time points

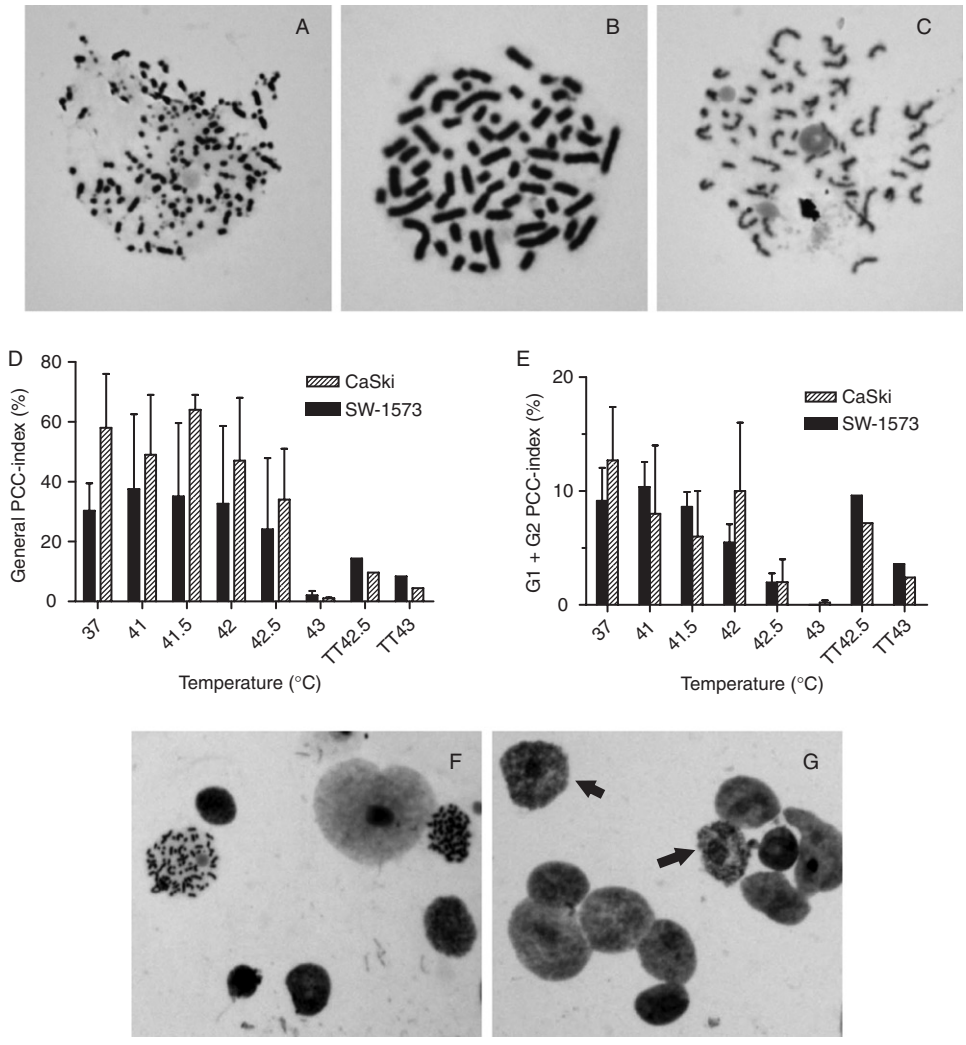

Figure 1. (A–C) Typical examples of S-phase (A), G2-phase (B) and G1-phase (C) PCC in SW-1573 cells. Magnification: 1,000 x. (D) Total PCC induction, and (E) induction of PCC in G1 and G2 phase cells by Cal in cells immediately after hyperthermia treatment at various temperatures for 1 h and in thermotolerant (TT) cells treated at the indicated temperature. Means with standard errors of three independent experiments. (F–G) Representative examples of SW-1573 nuclei after incubation of cells with Cal for 1 h to induce PCC. (F) untreated cells: two G2 phase PCC spreads and 6 non-condensed nuclei are visible, and (G) cells treated with 43°C hyperthermia (1 h), arrows: condensation of nuclei not resulting in chromosomal spreads. Magnification: 200 x.

after HT at 43°C (Figure 2). The results show that the inhibitory effect on PCC-induction persisted for 16 h after treatment in both cell lines. In addition, we observed that the inhibition of PCC-induction by HT at ≥42.5°C was reduced in cells, which were made thermotolerant by HT for 1 h at 41°C 6 h prior to the challenging HT treatment.

Phosphorylation status of Cdc2, H3 and Cyclin B1 does not correlate with PCC induction by Cal without or with HT at 43°C

Since dephosphorylation of Cdc2-P_{Tyr15} and phosphorylation of H3-P_{Ser10} are implicated in chromosome condensation [14–17], it was investigated whether HT at 43°C had an effect on the phosphorylation status of both proteins. As compared to control cells, dephosphorylation of Cdc2-P_{Tyr15} and phosphorylation of H3-P_{Ser10} was observed in SW-1573 cells treated with Cal similar to the levels found in mitotic cells (Figure 3). These changes in phosphorylation were also found in cells treated with Cal both directly and at 24 h after HT at 43°C. Total Cdc2 levels were slightly reduced 24 h after HT treatment at 43°C only. Similar results were obtained for CaSki cells (data not shown), suggesting that the overall phosphorylation status directly after HT at 43°C could not explain the observed inhibition of PCC-induction by Cal. Phosphorylated and total Cyclin B1 levels

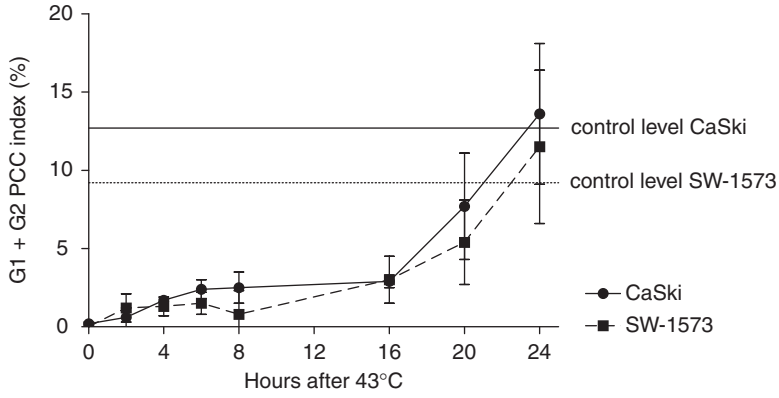

Figure 2. Induction of PCC in G1 and G2 phase cells after recovery at 37°C for various time periods after 43°C hyperthermia treatment. Means with standard errors of three independent experiments.

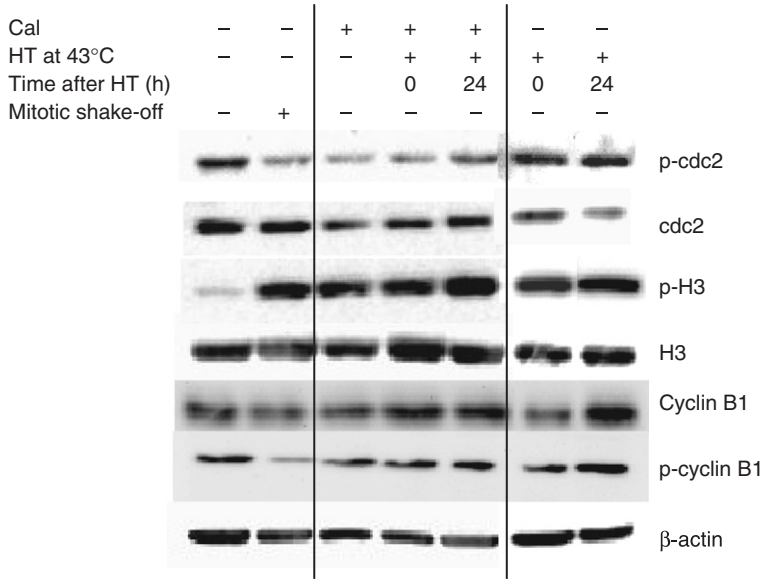

Figure 3. Representative example of two separate experiments of western blot analysis of the effect of treatment with Cal and/or HT at 43°C on Cdc2-P_{Tyr15}, total Cdc2, H3-P_{Ser10}, total H3, p-cyclin B1, total cyclin B1 and β-Actin in SW-1573 cells.

were not influenced by any of the treatments (Figure 3).

Cell cycle dependent phosphorylation of H3-P_{Ser10} by Cal is not influenced by HT at 43°C

To establish whether HT at 43°C influenced the phosphorylation status of H3-P_{Ser10} on a single cell basis, bivariate flow cytometric analysis was performed. In untreated CaSki cultures, most of the cells were in G0/G1 without H3-P_{Ser10}-positivity (Figure 4A). In mitotic cell suspensions obtained by shake-off, a tetraploid (4N) H3-P_{Ser10} positive mitotic population was clearly visible (Figure 4B). When cells were incubated for 1 h with Cal, the total amount of H3-P_{Ser10} positive cells (both 2N, G0/G1 phase and 4N, G2/M phase) was increased (Figure 4C). Treatment of cells for 1 h with HT at 43°C did not affect Cal-induced H3-P_{Ser10} (Figures 4D and E). The quantification of 2N and 4N of both CaSki and SW-1573 cells positive for H3-P_{Ser10} are shown in Figures 4F and G.

Discussion

In the present study we show that HT for 1 h transiently inhibits PCC induction by Cal at temperatures above 42.5°C. Apparently there is a temperature threshold for inhibition of proteins or structures

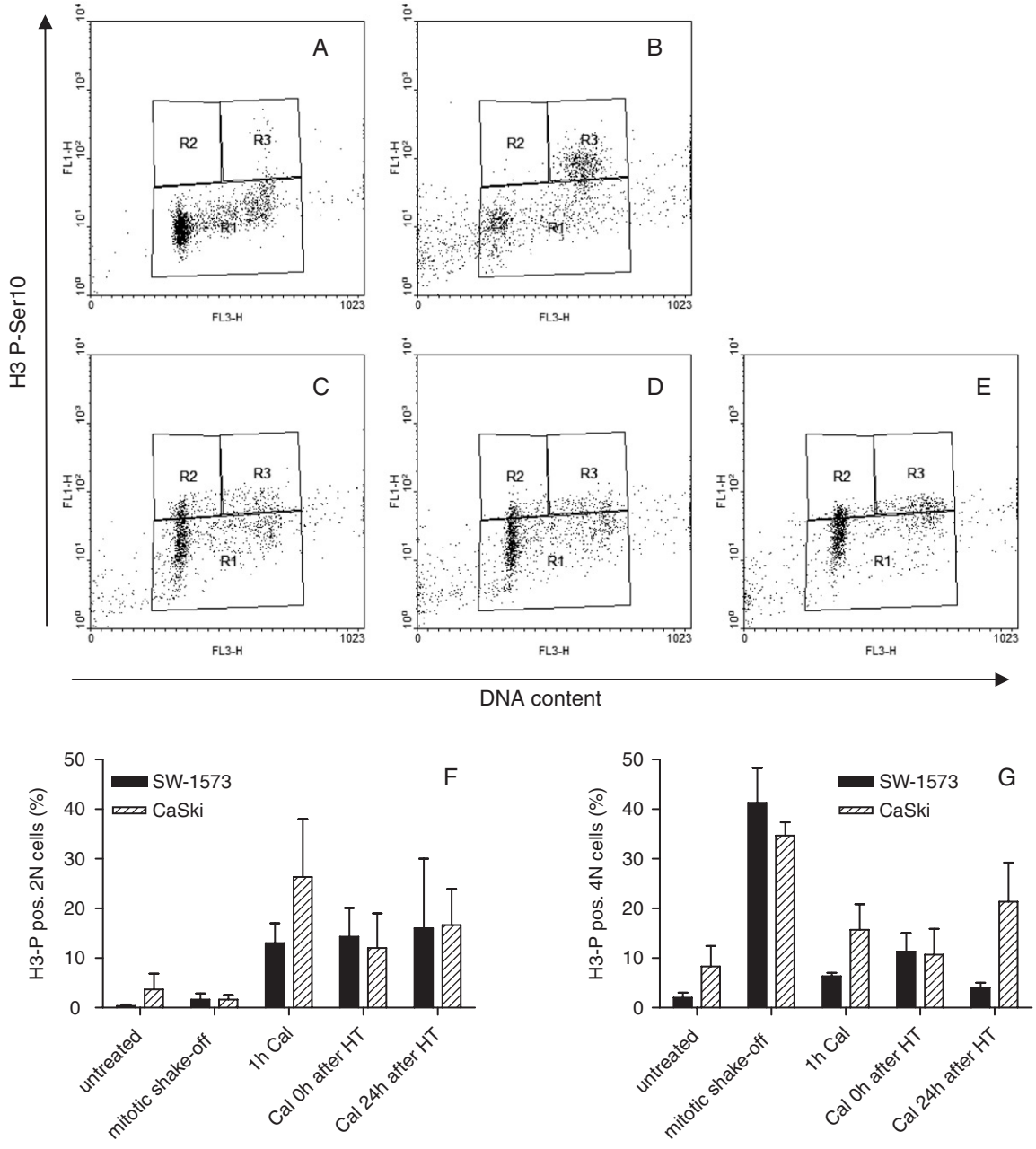

Figure 4. Phosphorylation of H3-P_{Ser10} is not associated with Cal induced PCC inhibition by 1 h hyperthermia treatment at 43°C. Representative examples of three independent experiments are shown for CaSki cells (A–E) y-axis: H3-P_{Ser10}fluorescence, x-axis: DNA content propidium iodide fluorescence; (A) Untreated, (B) mitotic shake-off cell suspension, (C) 1 h Cal, (D) Cal 0 h after HT at 43°C, (E) Cal 24 h after HT at 43°C. Regions are defined as H3-P_{Ser10} negative (R1), H3-P_{Ser10} positive 2N (R2) and H3-P_{Ser10} positive 4N (R3). Quantification of H3-P_{Ser10} positive 2N (F) and 4N (G) CaSki and SW-1573 cells.

important for PCC induction by Cal. Inhibition of PCC induction by the PEG-mediated cell fusion technique after HT at 43°C and at 45.5°C has been reported earlier for Chinese hamster ovary cells [24]. The ability of chromatin to condense by fusion with mitotic (unheated) cells decreased with increasing heating time and reached a plateau after 20 min of heating at 45.5°C. HT at 43°C totally inhibited induction of PCC after a 70 min heating period [24].

Our data indicate that the inhibition of PCC induction by HT at 43°C was transient in both cell lines. The recovery of PCC index was observed between 16 and 20 h and was complete at 24 h after HT. These findings are in line with the results of Iliakis et al. [24], who observed a recovery period for PCC index of 12 h after HT at 45.5°C for 8 min and over 24 h after HT at 45.5°C for 15 min. The recovery of PCC index over time might be

related to the expression of heat shock proteins that protect proteins from denaturation by heat [31, 32]. This hypothesis is underscored by our observation that the inhibition of PCC-induction by HT above 42.5°C was reduced in thermotolerant cells. A similar recovery time of heat-induced changes in chromatin structure and enzyme activities of 12–24 h after HT treatment ≥45°C, for 15–17 min has been reported by several others [33–35]. However, the induction of heat shock proteins and of thermo-tolerance has been reported to occur earlier [31, 36] than the recovery of the PCC index. Therefore, mechanisms other than thermotolerance must play a role.

Nuclear proteins appear to be particularly sensitive to HT-induced protein denaturation [37–40]. Denaturation of thermolabile proteins results in the aggregation and biological inactivation of important proteins [28], affecting important processes like DNA repair and synthesis [41], and inducing chromatin alterations [27]. Since dephosphorylation of the Tyr15 residue of Cdc2 has been associated with activation of the MPF and entry of cells into mitosis and/or chromosomal condensation [11 14, 42], we reasoned that HT at 43°C would interfere with this process. However, our results indicate Cdc2 phosphorylation is not associated with artificial chromosome condensation. This may be explained by observations that entry of cells into mitosis do not require Cdc2 activity [43]. The authors suggest that other proteins, such as histone H1 [44] and Cdc25C (independent from its function in Cdc2 kinase activation), might also be involved in chromosome condensation and G2/M transition.

A further downstream event in chromosome condensation is the phosphorylation of H3-P_{Ser10}, which has been associated with PCC induction after incubation of cells with Cal [18–20]. Cells treated with Cal alone showed H3-P_{Ser10} positive cells in all phases of the cell cycle, which explains the occurrence of G1, S as well as G2 phase PCCs in our study and is in agreement with others [19]. Surprisingly, however, we could not find an effect of HT at 43°C on the phosphorylation status of H3-P_{Ser10} in whole cell lysates nor in single-cell-based flow cytometry. Phosphorylation of H3-P_{Ser10} has been found to occur at transcriptionally activated heat shock loci implying a role for H3-P_{Ser10} in the regulation of gene expression in addition to its role in chromosome condensation [17]. However, H3-P_{Ser10} phosphorylation associated with transcriptional activation after heat has been shown to be a rapid and transient event and affects a cell population distinct from that normally detected in mitotic cells [45]. In contrast to transcription-associated H3-P_{Ser10} phosphorylation, condensed chromosomes exhibit very strong H3-P_{Ser10} right across chromosomes [46].

The appearance of some condensed nuclei after Cal treatment preceded by HT indicates that a certain level of DNA condensation can take place without formation of chromosomal spreads. This suggests that a late stage in the process of PCC is inhibited by HT. Iliakis et al. suggested that heat-induced alterations in the chromatin, nucleolus and the nuclear membrane might be involved in PCC inhibition as these structures serve as substrates for enzymatic reactions necessary for PCC [23]. According to our results, the effect of HT should be downstream of the phosphorylation of H3-P_{Ser10}. The HT-induced aggregation of proteins in the nuclear matrix can result in inhibition of DNA replication [38]. This might be another explanation for inhibition of PCC by HT as a recent study shows the tight coupling of chromosome condensation to DNA replication by drug-induced PCC [47].

In summary, the analysis of chromosomal aberrations in tumor biopsies from patients is currently feasible and has the potential to predict radiation treatment outcome both alone and in combination with HT at temperatures reached in the clinic [1, 2, 23]. In this report we show that HT for 1 h at temperatures ≥42.5°C inhibited PCC induction by Cal. This inhibition was transient, as recovery to the control levels was complete in a period of 24 h. Therefore, we conclude that an interval of at least 24 h after HT should be applied, when tumor biopsies are taken from patients treated with temperatures near or above 42.5°C.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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