Decrease in MAP3Ks expression enhances the cell death caused by hyperthermia

Atsushi Enomoto\textsuperscript{a}, Takemichi Fukasawa\textsuperscript{a,b}, Hiroshi Terunuma\textsuperscript{c}, Keiichi Nakagawa\textsuperscript{d}, Ayumi Yoshizaki\textsuperscript{b}, Shinichi Sato\textsuperscript{b} and Kiyoshi Miyagawa\textsuperscript{a}

\textsuperscript{a}Laboratory of Molecular Radiology, Center for Disease Biology and Integrative Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan; \textsuperscript{b}Department of Dermatology, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan; \textsuperscript{c}Medical Oncology, Tokyo Clinic, Tokyo, Japan; \textsuperscript{d}Comprehensive Radiation Oncology, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

\textbf{ABSTRACT}

\textbf{Purpose:} Hyperthermia is a promising anticancer treatment modality. However, the molecular mechanism underlying the thermal sensitivity of tumor cells is largely unknown. The aim of this study was to clarify how biochemical changes triggered by heat stimulate antitumor activity.

\textbf{Methods and materials:} The expression levels of various MAPK members in HeLa cells with or without hyperthermia were evaluated by western blotting and RT-PCR. The intracellular Ca\textsuperscript{2+} concentration [Ca\textsuperscript{2+}]\textsubscript{i} was monitored by digital imaging using Ca\textsuperscript{2+}TM-2 AM. An in vitro cleavage assay was used to determine whether calcium-dependent protease calpain cleaves MAPK components. Cell proliferation and clonogenicity were assessed in the absence or presence of siRNAs targeting MAPK members.

\textbf{Results:} Hyperthermia decreased the levels of MAP3K TAK1, RAF1 and MEKK2 but not of the downstream MAP2K and MAPK members. The hyperthermia-induced degradation of TAK1 and MEKK2 was rescued by either the proteasome inhibitor MG132 or the calpain inhibitor ALLN; however, RAF1 was not affected by the inhibitors. Heat induced down regulation of RAF1. Hyperthermia increased [Ca\textsuperscript{2+}]\textsubscript{i} and calpain I expression. The calcium ionophore A23187 decreased TAK1 and MEKK2 levels. An in vitro cleavage assay demonstrated that TAK1 and MEKK2 are calpain I substrates. Knockdown of TAK1, RAF1 and MEKK2 suppressed cell proliferation and clonogenicity.

\textbf{Conclusions:} Hyperthermia decreased the levels of MAP3K TAK1, RAF1 and MEKK2, without reduction of the downstream components in the MAP3K-MAP2K-MAPK cascade, by a calpain-dependent degradation pathway or transcriptional regulation. TAK1, RAF1 and/or MEKK2 play crucial roles in cell proliferation and clonogenicity and are potential molecular targets for hyperthermia.

\section*{Introduction}

Hyperthermia is a well-known method for cancer treatment and is often used in combination with radiotherapy or chemotherapy. Hyperthermia increases the cell temperature and induces many biochemical changes, such as the generation of reactive oxygen species, an increased intracellular calcium ion concentration and protein degradation [1,2]. Hyperthermia-induced protein denaturation, aggregation, or degradation is a key event in the disruption of cellular homeostasis [3,4]. Intracellular protein degradation is regulated by multiple proteolytic pathways, including lysosome-, calcium- and proteasome-dependent mechanisms [5,6]. Hyperthermia induces the proteasomal degradation of anti-apoptotic regulators and DNA repair proteins [7,8]. However, the molecular mechanisms underlying thermal protein degradation and their roles in thermal killing are largely unknown.

Mitogen-activated protein kinase (MAPK) signaling pathways mediate cellular responses to various extracellular stimuli, including growth factors and environmental stresses [9]. The MAPK pathway features a three-kinase cascade, including MAP3Ks (e.g., ERK and JNK), MAPK kinases (MAP2Ks; e.g., MEK and MKK) and MAPKK kinases (MAP3Ks; e.g., TAK1, RAF and MEKK). MAP3Ks phosphorylate and activate MAP2Ks, which in turn phosphorylate MAPKs. Increasing evidence from biochemical and genetic analyses suggests that MAP3Ks link various extracellular stimuli to cytoplasmic and nuclear effectors by activating downstream MAPK pathways [10]. Twenty-one MAP3Ks have been shown to activate the known MAP2Ks. TAK1 was originally found to function in transforming growth factor-\beta (TGF-\beta)-mediated MAPK activation [11]. TAK1 is also activated by a wide range of cytokines, such as IL-1 and TNF-\alpha [12]. Activated TAK1 then phosphorylates IKK and MKK, leading to the activation of NF-\kappaB and JNK [12,13]. RAF1 belongs to the RAF family of...
proto-oncogenes and is a component of the RAF/MEK/ERK signaling cascade [14]. RAF1 is not only related to oncogenesis but also plays important roles in cell growth and development [15,16]. MEKK2 activates the MEK5/ERK5 module and mediates epidermal growth factor (EGF) receptor and fibroblast growth factor-2 (FGF-2) receptor signals [17,18].

In this study, we evaluated the effect of hyperthermia on the expression of MAP3K members, including TAK1, RAF1 and MEKK2, as well as MAP2Ks and MAPKs. Based on our findings, we further investigated the molecular mechanisms underlying the hyperthermia-induced suppression of MAP3Ks and their impact on cell proliferation and clonogenicity.

**Materials and methods**

**Cell culture and stimulation**

HeLa cells were purchased from the Japanese Collection of Research Biosources Cell Bank (Ibaraki, Osaka, Japan). These cells were cultured in Dulbecco’s modified Eagle’s medium/F-12 (1:1) (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Hyclone, South Logan, UT) and 1% penicillin/streptomycin. LU99 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Hyperthermic treatment was carried out by submerging the culture flask in a water bath (Ikemoto Rika, Tokyo, Japan) set at temperature 41–45 °C or by inserting a catheter equipped with an 8-MHz capacitive heating device (Thermotron RF-8; Yamamoto Vinita Co., Ltd., Osaka, Japan) into the culture flask with an average power of 4.3–5.1 W. The indicated temperature was maintained with a precision of ±0.1 °C in the flask using a micro thermo probe IT-18 (Physitemp, Clifton, NJ). HeLa cells were pretreated with MG132 (Sigma, St. Louis, MO), ALLN (Sigma) or calpeptin (Calbiochem, Darmstadt, Germany) at 5–10 μM for 60 min and then subjected to hyperthermia for the indicated durations. To investigate the effect of calcium ionophore on the stability of MAP3Ks, HeLa cells were treated with 5 μM A23187 in PBS containing 1 mM CaCl2 (Cayman Chemical, Ann Arbor, MI) or vehicle for the indicated time periods. HeLa cells were treated with 40 μg/mL cycloheximide (CHX; Sigma) to inhibit protein synthesis. CHX-treated cells were harvested at the indicated times and processed for western blot analysis. To compare the cellular stress response to hyperthermia, the cells were irradiated using an X-ray generator (Pantak HF 350; Shimadzu, Kyoto, Japan) operating at 200 kV–20 mA, with a 0.5 mm Cu and 1 mm Al filter, at a dose rate of 1.33 Gy/min and 46 cm FSD. To measure the clonogenicity of MAP3K-knockdown cells, HeLa cells were transiently transfected with either a non-targeting small interfering RNA (siRNA) or a triple combination of TAK1-, MEKK2- and RAF1-specific siRNAs. At 24 h after transfection, the cells were trypsinized, diluted, counted and seeded on 60 mm dishes at various cell densities. After 14 d, the colonies were stained with crystal violet, and those containing more than 50 cells were counted. Cell proliferation was assessed by the ATP-based luminescence method using CellTiter-Glo® 2.0 Cell Viability Assay Kit (Promega, Madison, WI).

**Western blot analysis**

Equivalent amounts of total cell lysates were separated by SDS-PAGE and then transferred onto Immobilon PVDF membranes (Millipore, Bedford, MA). The membranes were blocked by Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 2% nonfat dry milk or Blocking One-P (Nacalai Tesque, Kyoto, Japan) for phosphoprotein detection. The blots were then incubated with one of the following antibodies: anti-TAK1/MAP3K7 (Proteinitech, Rosemont, IL), anti-MEKK2 (Epitomics, Burlingame, CA), anti-c-RAF (Cell Signaling Technology, Beverly, MA), anti-phospho-RAF1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-MEK1/2 (Cell Signaling Technology), anti-MEKK5 (Proteinitech), anti-SEK1/MKK4 (Cell Signaling Technology), anti-phospho-SEK1 (Cell Signaling Technology), anti-ERK1/2 (C-9, Santa Cruz Biotechnology), anti-ERK5/MAPK7 (Proteinitech), anti-JNK (Cell Signaling Technology), anti-Calpain I (GeneTex, Irvine, CA), anti-GST (Santa Cruz Biotechnology), anti-CDK2 (Santa Cruz Biotechnology), anti-GAPDH (MBL, Nagoya, Japan) or anti-β-actin (Sigma). The blots were washed thrice with TBS-T and incubated with secondary peroxidase-conjugated antibodies (Dako, Glostrup, Denmark). The resulting signals were detected on X-ray films (GE Healthcare, Buckinghamshire, UK) using an enhanced chemiluminescence detection system (GE Healthcare). The captured images were analyzed using ImageJ (NIH, Bethesda, MD) and quantified by measuring the density of each protein band.

**Ca2+ imaging**

HeLa cells were loaded with CaTM-2 AM (Goryo Chemical Inc., Sapporo, Japan), according to the manufacturer’s instructions. Briefly, HeLa cells were incubated in 3 μM CaTM-2 AM solution (1 mM CaTM-2 AM diluted in 20 mM HEPES buffer) at 37 °C for 60 min, rinsed with 20 mM HEPES buffer and kept in this buffer for de-esterification at room temperature for 30 min. After CaTM-2 AM loading, the cells were treated with heat, X-ray irradiation, or A23187 for the indicated times or left untreated. For Ca2+ imaging, a ZOE Fluorescent Cell Imager (Bio-Rad, Hercules, CA) was used with a red fluorescence filter.

**In vitro proteolytic cleavage assay**

The cleavage reactions were initiated by the addition of 0.035–0.14 units of calpain I (Calbiochem) to 200 ng of the purified GST-tagged TAK1 or MEKK2 in a total volume of 20 μL of the cleavage buffer (20 mM HEPES, pH 7.5, 50 mM KCl, 2 mM MgCl2, 5 mM CaCl2 and 1 mM DTT). The cleavage reactions were carried out for 30 min at 30 °C in a heat block and were stopped by the addition of 2 × SDS-sample buffer. The cleaved products were then analyzed by western blotting using an anti-GST antibody.

**In vitro kinase assays**

For in vitro kinase assays, immunoprecipitated TAK1 or MEKK2 was incubated with 1.0 μg of wild-type inactive MKK4
(Signal Chem, Richmond, BC) in kinase buffer (20 mM Tris-HCl [pH 7.5], 10 mM MgCl₂, 1 mM DTT) for 30 min at 30 °C. The kinase reaction products were subjected to SDS-PAGE and western blotting using an anti-phospho-MKK4 antibody. For in vitro MEKK2 autophosphorylation assays, GST-MEKK2 was incubated in kinase buffer containing 0.37 MBq mL⁻¹ [γ-³²P]ATP for 30 min at 30 °C. The kinase reactions were stopped by the addition of 2× SDS sample buffer, separated by SDS-PAGE, and analyzed using a phosphoimaging device (BAS-2500; FujiFilm, Tokyo, Japan).

**siRNAs and transfection**

Synthetic siRNA duplex oligonucleotides specifically targeting regions in human TAK1 (MAP3K7), RAF1 (C-RAF) and MEKK2 (MAP3K2) mRNAs were designed and synthesized by Invitrogen (Carlsbad, CA). The target sequences were as follows (antisense sequence is shown): Stealth siRNA TAK1 #542, 5′-GCAACAGAGUAAUCUGACGUUUA-3′; Stealth siRNA TAK1 #544, 5′-CCUAGUACGUAGCUUUCGCUUA-3′; Stealth siRNA MEKK2 #662, 5′-GGAACUCGUGACGUAGUAAUCAU-3′; Stealth siRNA MEKK2 #663, 5′-CCAAUAACGAGUUGG

**Figure 1.** Hyperthermia decreases expression levels of TAK1, RAF1 and MEKK2. (A) HeLa cells were heated at 41–45 °C for 60 min and harvested. Cell lysates were prepared and subjected to western blot analysis with antibodies against the indicated proteins. (B) The activity of TAK1 or MEKK2 was measured using an immune complex kinase assay with the appropriate specific antibody. RAF1 activity was analyzed by western blotting with a phosho-RAF1 antibody. (C, D) HeLa cells were heated to 45 °C or irradiated with X-rays at 2–5 Gy and harvested at the indicated time. Cell lysates were prepared and subjected to western blot analysis with antibodies against the indicated proteins. CDK2 was used as a loading control. Relative levels of MAPK-related proteins were determined from the western blots using ImageJ. Data are presented as means ± standard deviations of three independent experiments.
UAAUCCAUU-3′; Stealth siRNA RAF1 #462, 5′-GGAGUAACAUC AGACAACUCUUAUU-3′; Stealth siRNA RAF1 #468 and 5′-CCAUAGAGA-CAUGAAAUCCAACAAU-3′. Transient transfections were performed using Lipofectamine 2000 (Takara Bio, Shiga, Japan), according to manufacturer’s instructions.

**Semi-quantitative RT-PCR analysis**

Total RNA was isolated from both untreated and hyperthermia-treated cells using the ISOGEN II RNA Isolation System (Nippongene, Tokyo, Japan). RNA was converted to cDNA using SuperScript III reverse transcriptase (Invitrogen) and oligo(dT)₁₂₋₁₈ (GE Healthcare). The cDNA was amplified by polymerase chain reaction (PCR) using PrimeSTAR DNA Polymerase (Takara Bio) and specific primers (Santa Cruz Biotechnology).

**Results**

**Heat treatment reduces MAP3K protein levels**

MAPK pathways are activated by a wide variety of extracellular stimuli, such as X-ray irradiation or cytokines [9]. Therefore, we examined the effects of heat on the expression of MAPK family members. In HeLa cells, the expression or activity of the MAP3Ks TAK1, RAF1 and MEKK2 decreased with increase in the temperature (41–45 °C) and the duration of hyperthermic treatment at 45 °C (Figure 1(A–C)). Notably, hyperthermia caused rapid decline in the expression of the
MAP3Ks at temperatures 43 °C or higher. The heat-induced reduction in the levels of MAP3Ks was also observed in LU99 cells (Supplementary Figure S1(A)). Heat treatment did not decrease the expression of MAP2Ks, including MEK1, MEK5 and MKK4, and transiently increased the protein levels of MAPKs, such as ERK1/2, ERK5 and JNK1/2. Unlike heat treatment, reduction in the levels of TAK1, MEKK2 or RAF1 proteins were not observed after X-ray irradiation (Figure 1(D)). These results indicate that hyperthermia induces the suppression of some MAP3Ks but not of MAP2Ks or MAPKs.

We next hypothesized that the hyperthermia-induced decreases in MAP3K expression may be due to the downregulation of gene expression or protein instability. We assessed the effect of heat on gene expression levels of MAP3Ks by semi-quantitative RT-PCR. As shown in Figure 2(A), hyperthermia prominently downregulated RAF1 mRNA in proportion to the duration of treatment. The mRNA expression levels of TAK1 and MEKK2 decreased slightly in response to heat treatment, with no effect on ERK1 mRNA levels.
Furthermore, we inhibited protein synthesis by treating the cells with CHX and measured the degradation rate of RAF1 using western blot analysis. Treatment of HeLa cells with 40 \( \mu \)g/mL CHX resulted in a significant decrease in the steady-state level of endogenous RAF1 within 1 h (Figure 2(B)).

Thermal stress also causes protein unfolding [3]. These unfolded proteins are either refolded via molecular chaperones or are broken down, if the protein structure cannot be rescued [1]. We examined the two potential pathways by which heat-induced degradation occurs, namely the ubiquitin–proteasome pathway and calpain degradation. The addition of MG132, a commonly used 26S proteasome inhibitor, partially suppressed the heat-induced degradation of TAK1 and MEKK2 (Figure 2(C)). Another calpain inhibitor, ALLN, also significantly reversed the heat-induced degradation of TAK1 and MEKK2. However, neither MG132 nor ALLN rescued the degradation of RAF1 after heat treatment. These findings suggested that heat-induced reduction in TAK1 and MEKK2 levels mainly occur via the protein degradation pathway; however, the reduction in the RAF1 level can be attributed to transcriptional downregulation and protein lability.

**Ca\(^{2+}\)**-dependent degradation of TAK1 and MEKK2

Hyperthermia triggers endoplasmic reticulum stress or alters the permeability of plasma membranes, resulting in calcium spikes [19,20]. Thus, we studied the effects of various stimuli, including hyperthermia, on the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(\text{i}\)). Changes in [Ca\(^{2+}\)]\(\text{i}\) are shown in Figure 3(A) and Supplementary Figure S2. The frequency of cells showing elevated [Ca\(^{2+}\)]\(\text{i}\) was higher after hyperthermic treatment at 43–44 °C for 20 min than for untreated cells. In the presence of A23187, a greater number of cells showed a much higher [Ca\(^{2+}\)]\(\text{i}\). However, an increase in [Ca\(^{2+}\)]\(\text{i}\) was not observed in X-ray-irradiated cells under our experimental conditions until 1 h after 5 Gy of X-ray irradiation (Figure 3(A), Supplementary Figure S2). The increase in [Ca\(^{2+}\)]\(\text{i}\) activates calcium-dependent protease calpain [21,22]. Thus, we examined the effect of extracellular stimuli on the expression of calpain I. Exposing HeLa cells to hyperthermia slightly increased calpain I expression (1.6-fold) (Figure 3(B)) but exposure to X-ray marginally attenuated its expression (Figure 3(C)). Furthermore, the calcium ionophore A23187 decreased the levels of TAK1 and MEKK2 (Figure 3(D)). The addition of calpeptin, a calpain-specific inhibitor, partially suppressed the A23187-induced degradation of TAK1 or MEKK2. These findings indicate that hyperthermia induces an increase in [Ca\(^{2+}\)]\(\text{i}\) and the expression of calpain I, suggesting that calpain activation stimulates the degradation of TAK1 and MEKK2.

### Cleavage of TAK1 and MEKK2 by calpain I

Calpains are calcium-activated neutral proteases that catalyze the cleavage of a wide variety of proteins, including enzymes, transcription factors and cytoskeletal proteins, in many mammalian tissues [6,23]. To determine whether calpain directly cleaves MAP3Ks, we incubated purified calpain I with N-terminal GST-tagged TAK1 or MEKK2. While TAK1 showed limited proteolysis by calpain I, the amount of full-length MEKK2 decreased significantly in a dose-dependent manner after the addition of calpain I (Figure 4(A)). MEKK2 forms a dimer, leading to its activation by transphosphorylation [24,25]. As shown in Figure 4(B), wild-type MEKK2 underwent autophosphorylation, but the incorporation of \(^{32}\)P by

![Figure 4. Cleavage of TAK1 and MEKK2 by calpain I. (A) In vitro TAK1 and MEKK2 cleavage assays. GST-tagged TAK1 or MEKK2 was incubated with different concentrations of calpain I (0.035, 0.07 and 0.14 units of calpain I in lanes 2–5, respectively) in the absence or presence of 10 \( \mu \)M calpeptin for 30 min at 30 °C. The in vitro reaction products were subjected to western blot analysis with an anti-GST antibody. Arrows indicate cleaved products of TAK1 or MEKK2. (B) In vitro MEKK2 autophosphorylation assay. GST-MEKK2 was incubated with different concentrations of calpain I (0.035, 0.07 and 0.14 units of calpain I in lanes 2–4, respectively) for 30 min at 30 °C. The in vitro cleavage reaction products were further incubated in the presence of \( \gamma ^{32}\)P ATP for 30 min at 30 °C. The kinase reaction products were subjected to SDS-PAGE and then visualized by autoradiography.](image-url)
wild-type MEKK2 significantly decreased in the presence of calpain I. These results suggest that calpain cleaves MEKK2, resulting in the decrease of MEKK2 autophosphorylation. The calpain-mediated cleavage of TAK1 or MEKK2 was completely blocked by calpeptin, indicating that TAK1 or MEKK2 is a substrate of calpain I.

Depletion of TAK1, RAF1 and MEKK2 suppresses cell proliferation and clonogenicity

Hyperthermia kills cells in a time- and temperature-dependent manner [1]. We investigated the kinetics of thermal cell killing using a colony formation assay. When HeLa cells were heated, the surviving fraction decreased exponentially in a temperature- and time-dependent manner (Figure 5(A), left and right panels). To clarify the biological significance of MAP3K degradation after hyperthermia, we examined the effects of MAP3K knockdown on cell proliferation and clonogenicity. Transient transfection with different triple combinations of siRNAs targeting TAK1 (designated #T1 and #T2), RAF1 (designated #R1 and #R2) and MEKK2 (designated #M1 and #M2); 24 h later, cell lysates were prepared and subjected to western blot analysis with antibodies against the indicated proteins. The cells transfected with NC siRNA or triple siRNA combination (si MAP3K-1:#T1#R1#M1 or si MAP3K-2:#T2#R2#M2) were assayed at 24–96 h after transfection for cell proliferation by ATP-based luminescence assay. Proliferation efficiency of the transfected cells was determined by colony formation assay. CDK2 was used as a loading control. Data are presented as means ± standard deviations of three independent experiments. Statistical significance was determined using the Student’s t-test (*p < .05; **p < .01; ***p < .005).

Figure 5. Depletion of TAK1, RAf1 and MEKK2 suppresses cell proliferation and clonogenicity. Survival curves for HeLa cells heated at different temperatures for various durations. HeLa cells were heated at 43–45 °C for 30 min (A, left panel) or at 44–45 °C for the indicated time periods (A, right panel) and then subjected to a colony formation assay. (B,C) HeLa cells were transfected with a non-targeting control (NC) or different triple combinations of siRNAs targeting TAK1 (designated #T1 and #T2), RAF1 (designated #R1 and #R2) and MEKK2 (designated #M1 and #M2); 24 h later, cell lysates were prepared and subjected to western blot analysis with antibodies against the indicated proteins. The cells transfected with NC siRNA or triple siRNA combination (si MAP3K-1:#T1#R1#M1 or si MAP3K-2:#T2#R2#M2) were assayed at 24–96 h after transfection for cell proliferation by ATP-based luminescence assay. Proliferation efficiency of the transfected cells was determined by colony formation assay. CDK2 was used as a loading control. Data are presented as means ± standard deviations of three independent experiments. Statistical significance was determined using the Student’s t-test (*p < .05; **p < .01; ***p < .005).
plating efficiency was markedly lower for MAP3K siRNA-expressing HeLa cells than for cells treated with control siRNA (Figure 5(C)). These results indicate that TAK1, MEKK2 and/or RAF1 play important roles in cell proliferation and survival after heat treatment.

**Discussion**

Our results indicate that hyperthermia decreases the expression levels of MAP3K members (i.e., TAK1, RAF1 and MEKK2) but not the downstream MAP2K and MAPK members (Figure 1). Although TAK1, RAF1 and MEKK2 have a serine-threonine kinase domain, they each belong to a different MAP3K cluster (TAK, RAF or MEKK) based on kinase domain homology. These differences among MAP3Ks may confer differences in protein structure, function and regulation. In fact, RAF1 was significantly downregulated at the transcriptional level in response to hyperthermia, while the reduced expression levels of TAK1 and MEKK2 in response to hyperthermia were mainly due to proteolytic degradation (Figure 2(A–C)).

Hyperthermia induces an increase in [Ca$^{2+}$], [20,21]. Our results indicate that hyperthermia, but not X-ray irradiation, increased not only [Ca$^{2+}$], but also calpain I expression (Figure 3(A–C) and Supplementary Figure S2). Moreover, we demonstrated that the calcium ionophore A23187 induces the degradation of TAK1 and MEKK2 and identified TAK1 and MEKK2 as calpain substrates by in vitro cleavage assays (Figures 3(D) and 4(A)). Together, these results suggest that the degradation of TAK1 and MEKK2 by hyperthermia is mediated, at least in part, by a calpain-dependent pathway. As shown in Figure 4, calpains catalyze limited proteolysis of target proteins. However, cleaved isoforms of TAK1 or MEKK2 were not detected in the full-length blots using cell lysates after heat treatment (Supplementary Figure S1(B)). On the other hand, TAK1 and MEKK2 are ubiquitinated for degradation [26,27]. The hyperthermia-induced degradation of TAK1 and MEKK2 was partially rescued by the addition of MG132 (Figure 2(B)). Thus, heat may also induce the degradation of TAK or MEKK2 via an ubiquitin-dependent pathway, thereby decreasing the cleaved forms to undetectable levels.

In this study, the simultaneous knockdown of TAK1, RAF1 and MEKK2 attenuated cell proliferation and decreased clonogenicity of HeLa cells (Figure 5(B,C)). The MAPK pathways feature a three-kinase cascade, including MAP3K, MAP2K and MAPK. Moreover, emerging experimental evidence suggests that MAP3Ks can function selectively or cooperatively to tune a response [28]. TAK1 is a pivotal activator of MAPK signaling in inflammatory, immune and stress responses [29]. TAK1 controls cell viability and inflammation by activating downstream effectors, such as NF-κB and JNK, suggesting that it promotes cell survival by regulating apoptosis [13,30]. RAF1 stimulates cell proliferation and promotes cell survival by inhibiting apoptosis [15,16]. MEKK2 is a component of the ERK5 cascade, which regulates T-cell function and cytokine gene expression [18,31,32]. In addition to their central roles in MAPK signaling, TAK1, RAF1 and MEKK2 can activate the NF-κB signaling pathway, which regulates inflammatory responses and cell survival [30,33–35]. Aberrant NF-κB activity plays an important role in tumorigenesis and acquired resistance to chemotherapy [36]. Thus, hyperthermia-induced MAP3K degradation may be incapable of transducing proliferative stimuli into activation signals or activating each downstream target through phosphorylation, leading to the inhibition of cell survival, anti-apoptotic signaling, or functions and thereby contributing to thermal killing (Figure 6). Silencing of TAK1, RAF1, MEK5 or ERK5 was demonstrated to enhance radiosensitivity [37–40]. Together with our results, it can be suggested that hyperthermia-induced inhibition of MAP3Ks may contribute toward explaining the mechanism underlying the increase in the efficacy of radiotherapy by hyperthermia, especially at temperatures 43°C or higher.

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References

[1] Roti Roti JL. Cellular responses to hyperthermia (40-46 degrees C): cell killing and molecular events. Int J Hyperthermia. 2008; 24(1):13–15.

[2] Hou CH, Lin FL, Hou SM, et al. Hyperthermia induces apoptosis through endoplasmic reticulum and reactive oxygen species in human osteosarcoma cells. Int J Mol Sci. 2014;15(10):17380–17395.

[3] Ahmed K, Zaidi SF, Mati-Ur-Rehman MR, et al. Hyperthermia and protein homeostasis: cytoprotection and cell death. J Therm Biol. 2020;91:102615.

[4] Luo GJ, Sun X, Hasselgren PO. Hyperthermia stimulates energy-proteasome-dependent protein degradation in cultured myotubes. Am J Physiol Regul Integr Comp Physiol. 2000;278(3):R749–R756.

[5] Ciechanover A. Proteolysis: from the lysosome to ubiquitin and the proteasome. Nat Rev Mol Cell Biol. 2005;6(1):79–87.

[6] Goll DE, Thompson VF, Li H, et al. The calpain system. Physiol Rev. 2003;83(3):731–801.

[7] Song X, Kim SY, Zhou Z, et al. Hyperthermia enhances mapatumumab-induced apoptotic death through ubiquitin-mediated degradation of cellular FLIP(long) in human colon cancer cells. Cell Death Dis. 2013;4(4):e577.

[8] van den Tempel N, Odijk H, van Holthe N, et al. Heat-induced BRCA2 degradation in human tumours provides rationale for hyperthermia-PARP-inhibitor combination therapies. Int J Hyperthermia. 2018;34(4):407–414.

[9] Chang L, Karin M. Mammalian MAPK kinase signalling cascades. Nature. 2001;410(6824):37–40.

[10] Cobb MH, Goldsmith EJ. How MAP kinases are regulated. J Biol Chem. 1995;270(25):14943–14946.

[11] Yamaguchi K, Shirakabe K, Shibuya H, et al. Identification of a member of the MAPKKK family as a potential mediator of TGF-beta signal transduction. Science. 1995;270(5244):2008–2011.

[12] Sakurai H. Targeting of TAK1 in inflammatory disorders and cancer. Trends Pharmacol Sci. 2012;33(10):522–530.

[13] Ninomiya-Tsuji J, Kishimoto K, Hiyama A, et al. The kinase TAK1 in apoptosis induced by hyperthermia and its enhancement by verapamil in U937 cells. Int J Radiat Oncol Biol Phys. 2001;49(5):1369–1379.

[14] Kesavan K, Lobel-Rice K, Sun W, et al. MEKK2 regulates the coordinate activation of ERK5 and JNK in response to FGF-2 in fibroblasts. J Cell Physiol. 2004;199(1):140–148.

[15] Stevenson MA, Calderwood SK, Hahn GM. Effect of hyperthermia (45 °C) on calcium flux in Chinese hamster ovary HA-1 fibroblasts and its potential role in cytotoxicity and heat resistance. Cancer Res. 1987;47(4):3712–3717.

[16] Kameda K, Kondo T, Tanabe K, et al. The role of intracellular Ca(2+) in apoptosis induced by hyperthermia and its enhancement by verapamil in U937 cells. Int J Radiat Oncol Biol Phys. 2001;49(5):1369–1379.

[17] Suzuki K, Hata S, Kawabata Y, et al. Structure, activation, and biology of calpain. Diabetes. 2004;53(1):512–518.

[18] Moldoveanu T, Hosfield CM, Lim D, et al. A Ca2+ switch aligns the active site of calpain. Cell. 2002;108(5):649–660.

[19] Campbell RL, Davies PL. Structure-function relationships in calpains. Biochem J. 2012;447(3):335–351.

[20] Enomoto A, Fukasawa T, Tsumoto H, et al. Prevention of calpain-dependent degradation of STK38 by MEK2-mediated phosphorylation. Sci Rep. 2019;9(1):16010.

[21] Cheng J, Lu Y, Zhang D, et al. Dimethylation across the catalytic domain is essential for MEK2 activation. J Biol Chem. 2005;280(14):13477–13482.

[22] Fan Y, Shi Y, Liu S, et al. Lys48-linked TAK1 polyubiquitination at lysine-72 downregulates TNFα-induced NF-κB activation via mediating TAK1 degradation. Cell Signal. 2012;24(7):1381–1389.

[23] Yamashita M, Ying SX, Zhang GM, et al. Ubiquitin ligase smurf1 controls osteoblast activity and bone homeostasis by targeting MEK2 for degradation. Cell. 2005;121(1):101–113.

[24] Cueva BD, Abell AN, Johnson GL. Role of mitogen-activated protein kinase kinase kinases in signal integration. Oncogene. 2007;26(22):3159–3171.

[25] Sato S, Sanjo H, Takeda K, et al. Essential function for the kinase MEK5 in innate and adaptive immune responses. Nat Immunol. 2005;6(11):1087–1095.

[26] Takaesu G, Surabhi RM, Park KJ, et al. TAK1 is critical for IkappaB kinase activation via targeting MEKK2 for degradation. Cell. 2005;121(1):101–113.

[27] Guo Z, Clydesdale G, Cheng J, et al. Disruption of Mekk2 in mice reveals an unexpected role for MEK2 in modulating T-cell receptor signal transduction. Mol Cell Biol. 2002;22(16):5761–5768.

[28] Garrington TP, Ishizuka T, Papst PJ, et al. MEKK2 gene disruption causes loss of cytokine production in response to IgE and c-Kit ligand stimulation of ES cell-derived mast cells. EMBO J. 2000;19(20):5387–5395.

[29] Karin M, Ben-Neriah Y. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. Annu Rev Immunol. 2000;18:621–663.

[30] Zhao Q, Lee FS. Mitogen-activated protein kinase/ERK kinase kinases 2 and 3 activate nuclear factor-κB through IκBα and IκB kinase-beta. J Biol Chem. 1999;274(13):8355–8358.

[31] Schmidt C, Peng B, Li Z, et al. Mechanisms of proinflammatory cytokine-induced biphasic NF-κB activation. Mol Cell. 2003;268(1):105–115.

[32] Xia Y, Shen S, Verma IM. NF-κB, an active player in human cancers. Cancer Immunol Res. 2014;2(9):823–830.

[33] Furusawa Y, Wei ZL, Sakurai H, et al. TGF-β activates nuclear factor-κB through IκBα and IκB kinase-beta. J Biol Chem. 1999;274(13):8355–8358.

[34] Schmidt C, Peng B, Li Z, et al. Mechanisms of proinflammatory cytokine-induced biphasic NF-κB activation. Mol Cell. 2003;268(1):105–115.

[35] Leicht DT, Balan V, Kaplun A, et al. Raf kinases: function, regulation and role in human cancer. Biochim Biophys Acta. 2007;1773(8):1196–1212.

[36] Mikula M, Schreiber M, Husak Z, et al. Embryonic lethality and fetal liver apoptosis in mice lacking the c-Raf-1 gene. EMBO J. 2001;20(8):1952–1962.

[37] Kebach S, Ash J, Amnis MG, et al. Grb10 and active RAF-1 kinase promote bad-dependent cell survival. J Biol Chem. 2007;282(30):21873–21880.

[38] Blank JL, Gerwins P, Elliott EM, et al. Molecular cloning of mitogen-activated protein/ERK kinase kinases (MEKK) 2 and 3. Regulation of sequential phosphorylation pathways involving mitogen-activated protein kinase and c-Jun kinase. J Biol Chem. 1996;271(10):5361–5368.

[39] Kesavan K, Lobel-Rice K, Sun W, et al. MEKK2 regulates the coordinate activation of ERK5 and JNK in response to FGF-2 in fibroblasts. J Cell Physiol. 2004;199(1):140–148.