Protective effects of thioredoxin-mediated p53 activation in response to mild hyperthermia

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Abstract. Recently, mild hyperthermia was shown to induce cell cycle arrest at the G_2/M phase transition without leading to DNA damage. The mechanism of this regulation has not yet been elucidated, although p53 has been shown to be activated in response to mild hyperthermia. Here, we report the role of thioredoxin (TXN) in mild hyperthermia-induced cellular responses. Our data showed that the protein levels of p53 and its downstream gene, Gadd45a, which is an indicator of G_2/M arrest, were significantly decreased in TXN siRNA-treated cells under conditions of mild hyperthermia (41˚C, 60 min) as compared to TXN wild-type cells, implying that TXN might play an important role in mild hyperthermia-induced G_2/M arrest via p53 and Gadd45a activation. Furthermore, the release of cyclin-dependent kinase Cdc2, known to be regulated by Gadd45a under G_2/M arrest, was inhibited from the nucleus for arrest in the G_2/M phase in TXN downregulated cells under mild hyperthermia. We suggest that G_2/M arrest mediated via the TXN-modulated p53 in response to mild hyperthermia may provide critical insight into the clinical use of mild hyperthermia to induce an adaptive response against genotoxic stresses.

Introduction

An adaptive response to mild hyperthermia was first observed in *E. coli* by Cairns and his collaborators, who showed that *E. coli* can acquire transient resistance to a subsequent toxic dose of alkylating agents after sublethal treatment with an alkylating agent (1). The adaptive responses of the cells to other types of stress have significant implications to the potential prevention of DNA damage induced by various genotoxic stresses. Adaptive responses induced by heat stress might be applicable to a clinical approach to cancer. Recently, due to the characteristics of G_2/M arrest induced by the non-genotoxic conditions of mild hyperthermia, its clinical application has been reported. Whole-body hyperthermia combined with anti-cancer drugs (2) has shown benefits in both the control of local recurrence and improvement in patient survival (3). Despite these studies, the mechanisms involved in the cellular responses to mild hyperthermia have not been clarified yet.

The p53 protein is activated by non-genotoxic conditions such as hypoxia and oncogene activation as well as by a wide range of agents that induce genotoxic damage such as UV irradiation, carcinogens, and oxidative stress (4,5). Recently, hyperthermia has been shown to induce p53 activation as a non-genotoxic stress. Most studies have focused on the observation that hyperthermia treatment induces p53-dependent apoptosis in tumor cells (6,7). In addition, combined hyperthermia treatment with anti-cancer agents has shown to increase the sensitivity of tumor cells to such drugs. However, the mechanism behind the protective effect of hyperthermia as a non-genotoxic stress in normal cells has not yet been elucidated. Indeed, p53 responses to heat stress are less characteristic, although p53 responses to DNA damage have been extensively investigated.

Generally, the regulation of p53 activation has been shown to involve post-transcriptional modification (8), such as phosphorylation (9), acetylation (10), and sumoylation (11,12). In addition, post-transcriptional modifications have been reported to play essential roles in p53 stabilization (13,14). On the other hand, p53 activation by oxidative/reductive (redox) modulation has also been reported (15). Our previous study suggested that p53 might be activated via modulation of its redox status through thioredoxin (TXN) and redox factor 1 (Ref-1). Activation of p53 might be mediated by redox-sensitive interactions between TXN and Ref-1 (16).

TXN has been demonstrated to be a central intracellular antioxidant as well as an important regulator of redox-sensitive gene expression (17-20). In addition, TXN has been shown to act as an electron donor for ribonucleotide reductase, thereby providing deoxyribonucleotides for DNA synthesis and repair. Indeed, a number of studies have reported that reduced intracellular concentrations of TXN cause chromosomal instability and skin hyperpigmentation (21,22).

Activated p53 is well known as a sequence-specific transcription factor capable of mediating its downstream effects through

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the activation of target genes such as p21, Bax, and Gadd45. These genes induce many cellular responses such as cell cycle arrest, apoptosis, differentiation, and inhibition of angiogenesis in order to protect cells from carcinogenic risks (23,24). One p53-regulated gene, Gadd45a, is known to be involved in the control of the G_2/M checkpoint as a stress-inducible protein (22), although the mechanisms of Gadd45-mediated cellular responses have yet to be explored. A recent study reported that Gadd45a might promote G_2/M arrest via nuclear export and via the kinase activity of Cdc2, a cell cycle regulator (25).

Here, we studied the mechanism driving mild hyperthermia-induced G_2/M arrest in human cell lines. Our study suggests that the TXN-dependent redox state might play an important role in mild hyperthermia-induced p53 activation and cell cycle arrest via the cellular modulation of Gadd45a and Cdc2.

Materials and methods

Cells and hyperthermia. Cell cultures were maintained in RPMI-1640 with 10% FBS plus antibiotics. Exponentially growing human colon RKO cells were heat-treated at 41˚C by immersion in a water bath (Precision Inc., Elk Grove Village, IL, USA) for 70 min. Ten minutes after the dishes had been placed in the water bath, the desired temperature in the dishes was reached, and this was taken as the starting time of the hyperthermic temperature treatment.

TXN siRNA transfection. siRNA duplexes targeting TXN were designed and synthesized by Ambion Inc. (Austin, TX, USA). Transfection of RKO was accomplished using Oligofectamine Reagent (Invitrogen GmbH, Karlsruhe, Germany) according to the manufacturer's instructions with single-strand sense and antisense RNA oligonucleotides (human TXN sense RNA: 5'-AUGACUGUCAGGAUGUUGCtt-3'; human TXN antisense RNA: 5'-GCAACAUCCUGACAGUCAUcc-3'). In each cell culture dish, 60 μl of 20 μM siTXN were diluted with 350 μl of Opti-MEM (Invitrogen). Then, 8 μl of Oligofectamine were diluted and incubated for 10 min in 22 μl of Opti-MEM. The 2 solutions were combined at room temperature for 15 min, and the mixture was used to transfect RKO cells. After 4 h of transfection, the medium was replaced with RPMI-1640. Cells were harvested after 36 h for Western blot analysis.

Cell cycle analysis

Fluorescence-activated cell sorter (FACS) analyses. RKO and TXN siRNA-treated RKO cells were treated under the conditions of mild hyperthermia (41˚C, 70 min) and then processed for flow cytometry as previously described (26). After 0, 24, and 36 h incubation, cells were fixed in 70% ethanol and stained with 50 μg/ml of propidium iodide. Flow cytometric profiles were determined using a Becton-Dickinson FACScan and analyzed with the Cell Fit software.

Mitotic Index. Cell samples for microscopic analysis were processed using standard cytogenetic methods. Briefly, cells were treated with 0.2 μg/ml of colcemid for 2 h before harvesting and centrifugation at 15,000 rpm for 5 min, followed by pre-heating with 0.075 M KCl for 30 min at 37˚C and fixation in 3:1 methanol: acetic acid solution. Cell suspensions were dropped onto glass slides and stained in 5% Giemsa for 25 min. To minimize variability throughout the study, the same scorer performed the mitotic index evaluations. The frequency of mitotic cells per culture condition was determined by tallying the number of mitotic cells out of a population of 1,000 consecutive cells, and by ignoring broken cells, clumped cells, and cellular debris. The percentages of mitotic cells were calculated by comparison with control fractions (no heat treatment).

Immunoblotting. The cells were suspended in RIPA buffer (50 mM Tris, pH 7.2, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.05% SDS) and were treated by freezing and thawing 3 times. The protein content of the supernatant obtained after centrifugation (15,000 x g) was quantified using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richomond, CA). Each aliquot of protein (20 μg) was subjected to Western blot analysis for p53 and Gadd45. After electrophoresis on 10% polyacrylamide gel containing 0.1% SDS, the proteins were transferred electrophoretically onto Poly Screen PVDF membranes (DuPont/Biotechnology Systems NEN Research Products, Boston, MA). The membranes were then incubated with anti-p53 monoclonal antibody (PAb421, Oncogene Science, Inc., Uniondale, NY), anti-TXN polyclonal antibody (FL-105, Santa Cruz), and anti-Gadd45a polyclonal antibody (AbH-165, Santa Cruz). For vizualization of the bands, we used horseradish peroxidase-conjugated anti-mouse IgG antibody (Zymed Labs. Inc., San Francisco, CA) and the BLAST®: Blotting Amplification System (DuPont/Biotechnology Systems NEN Research Products).

Immunostaining. Cells were fixed with iced 100% methanol for 30 min at -20˚C followed by removal of methanol. The cells were then dehydrated with iced 100% acetone for 1 sec and washed 3 times with PBS. Staining was performed by incubation with Cdc2 mouse antibody (BD Biosciences, USA) diluted 1:200 in BSA solution (0.5% bovine serum albumin in PBS) as the primary antibody for 4 h at room temperature. After the primary antibody incubation, coverslips were washed 4 times for 5 min each with PBS containing 0.1% Tween-20. The cells were then incubated with fluorescein-isothiocyanate (FITC)-conjugated anti-mouse secondary antibody (Vector Laboratories, USA) for 1 h at room temperature, followed by 6 washes for 5 min each with shaking. Coverslips were mounted on standard microscope slides with mounting solution containing DAPI (Vector Laboratories). Images were viewed and captured using a fluorescence microscope (NIKON ECLIPSE 50i and NIS-Elements F 2.20, Nikon, Japan).

Cell viability test. One-half milliliter of a suitable cell suspension (cells diluted in complete serum-free medium to an approximate concentration of $1x10^5$ to $2x10^5$ cells/ml) was placed in a screw cap test tube. Trypan blue stain (0.4%) was added to the cells and then mixed thoroughly. Stained cells were allowed to stand for 5 min at 15 to 30˚C (room temperature). Cell counting was performed using a hemocytometer.

Results

TXN-dependent alteration of p53 and Gadd45 expression under mild hyperthermia. To investigate whether or not TXN

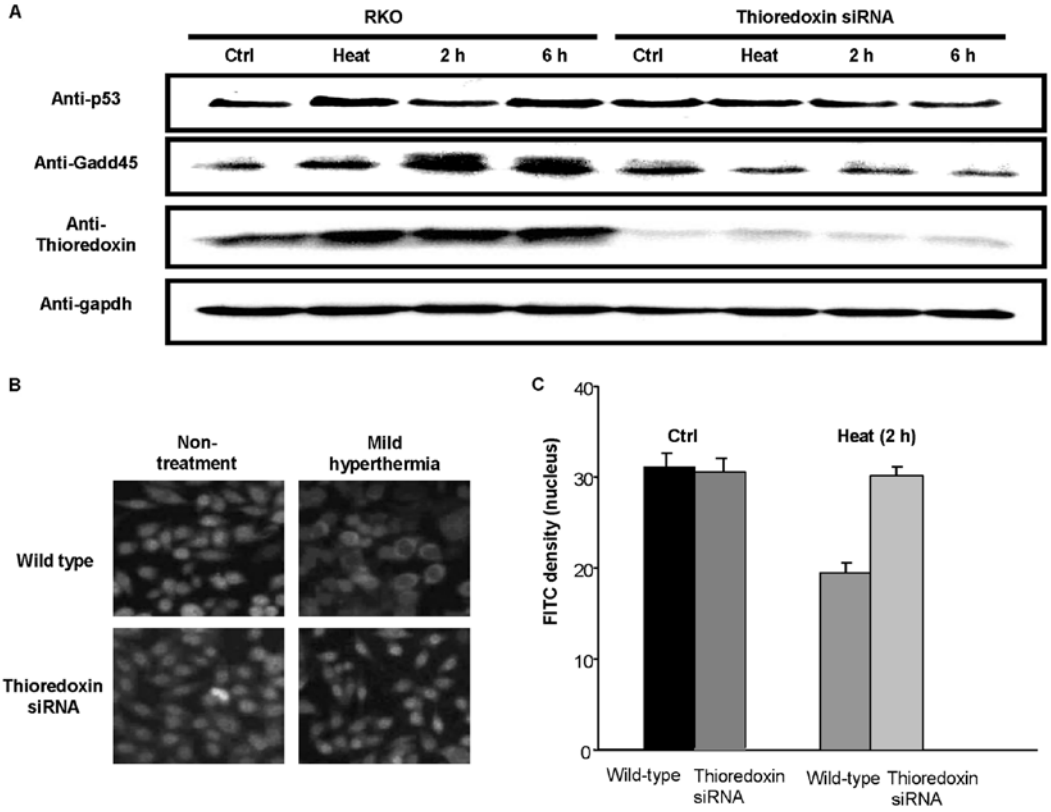

Figure 1. Decrease in p53 and Gadd45a expression and inhibition of Cdc2 import into the nucleus by TXN siRNA in response to mild hyperthermia. (A) Human colon RKO cells were transfected with TXN siRNA and incubated for 36 h. TXN protein levels of RKO cells were determined by Western blot analysis. Both p53 protein and Gadd45a expression were downregulated in TXN siRNA-treated RKO cells in contrast to TXN wild-type cells. (B) Inhibition of Cdc2 export from the nucleus by TXN siRNA treatment in response to mild hyperthermia. Images of stained nuclei were captured using a confocal microscope (x400 magnification, Carl Zeiss, Germany). Representative of 4 independent experiments. (C) Bar graphs indicate the fraction of Cdc2 in the nucleus in TXN wild-type cells and TXN siRNA-treated cells. Consistent data with the previous image analysis data are shown. The data represent the mean ± SD from at least 3 determinations. P<0.01 by t-test (SigmaPlot software, Systat Software, Inc., USA).

is involved in p53 activation in response to mild hyperthermia (41˚C for 60 min), we examined p53 protein accumulation in TXN wild-type cells and TXN siRNA-treated cells by Western blotting. The results showed an increase in p53 protein accumulation by mild hyperthermia in TXN wild-type cells as compared to TXN siRNA-treated cells (Fig. 1A). In addition, Gadd45, a p53-responsive stress protein, also showed increased levels of p53 accumulation in TXN wild-type cells than in TXN siRNA-treated cells, indicating that the TXN-mediated activation of p53 in response to mild hyperthermia involved Gadd45a activation (Fig. 1A).

Cdc2 localization. Cdc2 is known to be a nuclear-localized cell cycle regulator. G_2/M arrest is induced via a decrease in Cdc2 kinase activity and nuclear export of Cdc2. Our data showed that Cdc2 was localized to the nucleus in TXN siRNA-treated cells, in contrast to its cytoplasmic localization in TXN wild-type cells under mild hyperthermia, suggesting that TXN might be involved in G_2/M arrest via the nucleus export of Cdc2 in response to mild hyperthermia (Fig. 1B). These results were consistent with the quantification data of the fraction of stained Cdc2 in the nucleus (Fig. 1C).

Induction of G_2/M arrest in TXN wild-type cells under mild hyperthermia. We investigated the cellular responses to p53 and Gadd45 accumulation induced by mild hyperthermia using FACS analysis. Our data showed that the fraction of cells in the G_2/M phase was altered according to the presence or absence of TXN in RKO cells under mild hyperthermia. An increase in the fraction of cells in the G_2/M phase and a decrease in the S-phase fraction were induced in TXN wild-type RKO cells 24 h after heat treatment. In contrast, this shift was not observed in TXN siRNA-treated cells, suggesting that TXN might be an important mediator of G_2/M arrest induced by mild hyperthermia (Fig. 2A and B). Furthermore, the fraction of cells in sub-G_1 phase was significantly increased 36 h after mild hyperthermia treatment in TXN downregulated cells (Fig. 2C), indicating that TXN might be involved in cell survival under mild hyperthermia.

Alteration of mitotic index and cell viability in TXN downregulated cells. In addition, the mitotic index, which is a method for the quantification of G_2/M phase cells, was measured to confirm the induction of G_2/M arrest under mild hyperthermia. Our data showed significant inhibition of cell cycle progression in TXN wild-type cells in the presence of mild hyperthermia in contrast to TXN siRNA-treated cells also subjected to hyperthermia (Fig. 3A). Furthermore, our data showed that cell viability was significantly decreased in TXN siRNA-treated cells 36 h after mild hyperthermic treatment as compared to TXN wild-type cells (Fig. 3B).

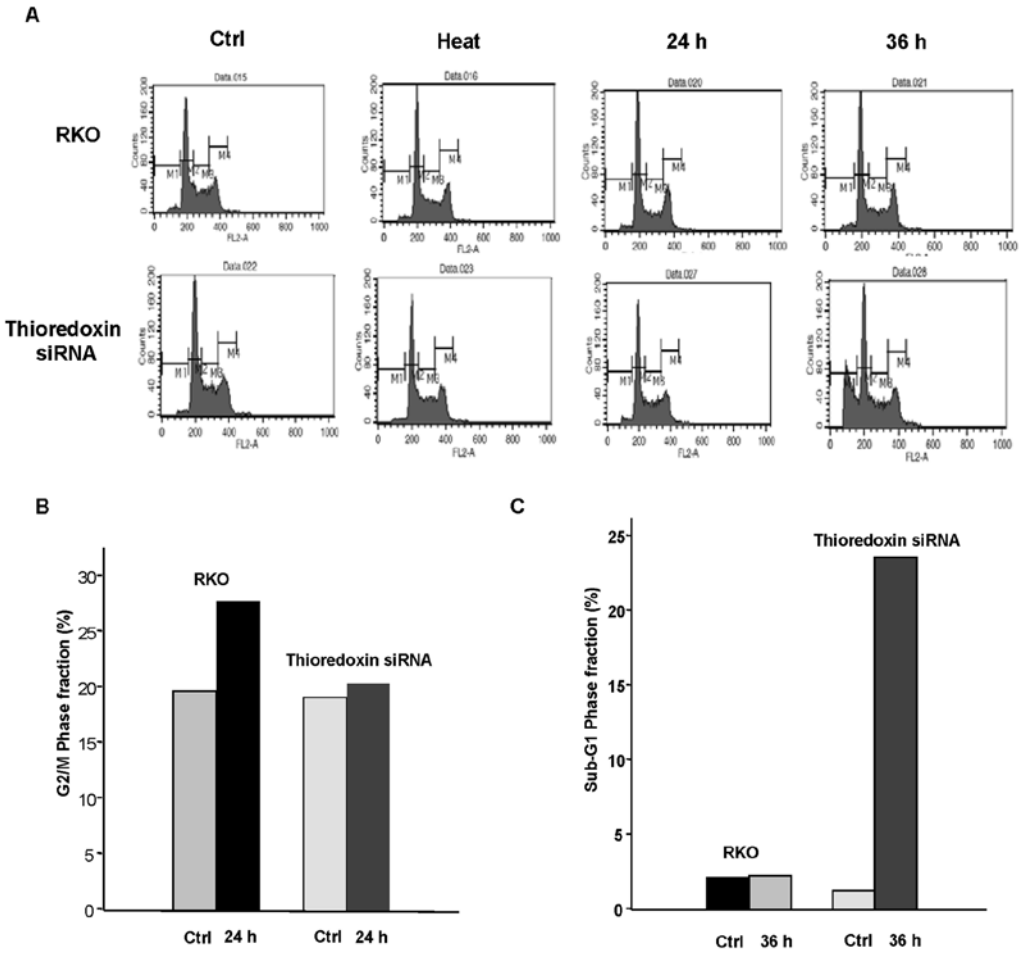

Figure 2. G_2/M arrest following DNA damage in TXN wild-type RKO cells. (A) Flow cytometric profiles shown by PI staining. In the absence of mild hyperthermia (41˚C for 70 min), cell cycle profiles were similar (44 to 47% G_1, 22 to 23% S, 17 to 22% G_2/M). After mild hyperthermia, TXN wild-type RKO cells exhibited marked G_2/M-phase delay. The results shown correspond to 24 h after heat shock. (B) Summary of cell cycle profiles in thiredoxin wild-type and blocked RKO cells in response to mild hyperthermia. The results shown in (A) are summarized by bar graphs. Again, TXN blocked cells were delayed in the G_2/M phase progression. Results shown correspond to 24 h after heat shock. (C) Sub-G_1 phase fraction was increased in TXN siRNA-treated cells. The data in (B and C) represent the mean ± SD from at least 3 determinations. $P<0.01$ by t-test (SigmaPlot software, Systat Software, Inc.).

Discussion

p53 is activated by various stresses, not only DNA damaging agents such as irradiation (27), carcinogens (28), and oxidative stress (29), but also non-DNA damaging agents such as hyperthermia (5), hypoxia/anoxia (30), and taxanes (29). Recently, the activation of p53 by non-genotoxic stress has been reported to lead to cellular protective effects, including induction of DNA repair pathways and cell cycle arrest, although the mechanisms of these protective effects induced by non-genotoxic agents require further study.

Pathways of p53 activation following stress signaling have been extensively investigated. Recently, the cellular redox status has been reported to be involved in p53 activation, in addition to mechanisms such as acetylation, sumoylation, and phosphorylation. One of these cytoplasmic and redox-sensitive signaling factors, TXN, has been shown to regulate nuclear transcription factors such as p53, AP-1, and NF-κB in mediating cellular responses to environmental stress (31). Especially in the presence of a reducing agent, TXN and Ref-1 have been shown to act as potent stimulators of wild-type p53 (32), although the involvement of TXN in p53 activation under non-genotoxic stress conditions such as hyperthermia has not yet been clarified.

In this study, we investigated the role of TXN in the regulation of p53 activation and cellular responses under the conditions of mild hyperthermia. Our previous study showed that p53 activation in response to mild hyperthermia (40˚C for 30 min) induces protective effects against a UV-mimetic agent, 4-nitroquinoline-1-oxide (4NQO) (33-35). On the other hand, the high temperature of hyperthermia (42-44˚C for 30 min) might induce apoptosis in cells as a protective response to DNA damage (Fig. 4). We used mild hyperthermia (41˚C for 60 min) to induce p53 activation without genotoxicity. As shown in Fig. 1, p53 levels were decreased in TXN siRNA-treated cells under mild hyperthermia in comparison to TXN wild-type cells, suggesting that p53 activation induced by mild hyperthermia might be modulated by a TXN-dependent redox state. Recent studies reported that the downregulation of endogenous normal TXN by siRNA induces the functional disruption of p53 (36), although the inhibition of TXN reductase is not enough to decrease p53 activity or expression without the inhibition of apurinic/apyrimidinic endonuclease 1/Redox factor 1 (APE1/Ref-1) (37). Our study focused on the role

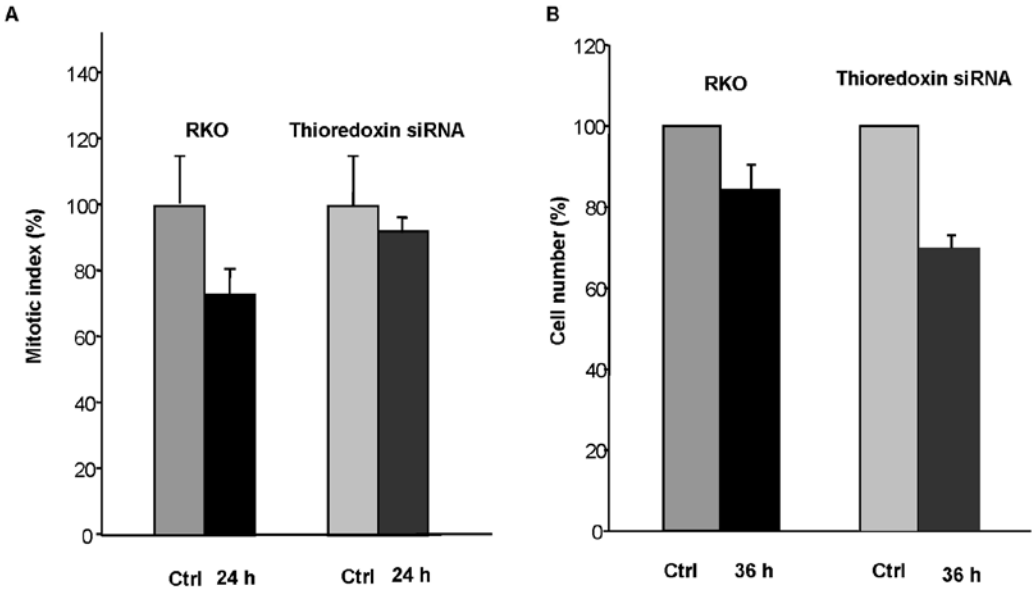

Figure 3. (A) Mitotic index shows significant inhibition of cell cycle under mild hyperthermia in TXN-downregulated RKO human cells. The data represent the mean ± SD from at least 3 determinations (P<0.01, t-test using the SigmaPlot software). Points, means of 4 replicates; bars, SD (B) Cell viability was significantly decreased by TXN siRNA 36 h after treatment with mild hyperthermia. The data represent the mean ± SD from at least 3 determinations (P<0.01, t-test using the SigmaPlot software).

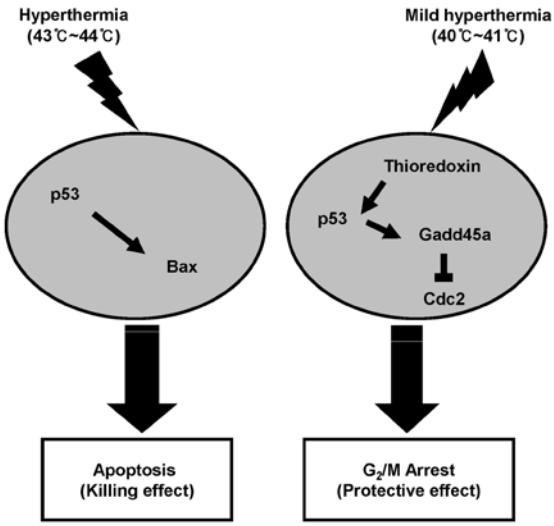

Figure 4. Suggested model for the mechanisms behind the cellular responses to mild hyperthermia. Hyperthermia is known to induce cellular responses such as apoptosis or G_2/M arrest according to the range of hyperthermia. Our group and other groups have reported that a high level of heat treatment from 43˚C to 44˚C might induce apoptosis in cells via activation of pro-apoptotic response genes, including p53 and its downregulated gene, Bax. On the other hand, this study suggests for the first time that mild hyperthermia between 40-41˚C might induce G_2/M arrest through TXN-mediated p53 and Gadd45a accumulation and export of Cdc2 from the nucleus. Clarification of G_2/M arrest under mild-hyperthermia might provide an important insight into the adaptive responses protecting cells from genotoxic stresses.

of endogenous TXN to investigate the mechanism behind the protective effects of mild hyperthermia in normal cells, although recent studies have shown that the overexpression of TXN might cause cancer cells to become resistant to anti-cancer drugs. Indeed, these evidences of TXN-mediated p53 activation in response to non-genotoxic stresses might support a role for p53 in inducing cellular responses such as cell cycle arrest and DNA repair in normal cells against carcinogens. However, the relationship between the p53 and overexpressed-TXN in cancer cells remains to be explored in future studies.

Distinct regulation mechanisms of p53 activation may induce different cellular responses according to the activation of particular downstream genes, such as p21, Bax, and Gadd45a. Recent studies have shown that one p53 target gene, Gadd45a, plays an important role in the maintenance of genomic stability in response to genotoxic agents. Hollander and his colleagues have reported that Gadd45a deletion has a substantial impact on genomic instability and growth control (38). In particular, Gadd45a has been shown to be a stress-inducible protein involved in the control of the G_2/M checkpoint (Fig. 4). Furthermore, human cells reduced in Gadd45a expression by anti-sense Gadd45a fail to arrest at G_2/M phase after exposure to either UV radiation or methylmethane sulfonate (39). Our data showed that the level of Gadd45a was increased under mild hyperthermia in TXN siRNA-treated cells as compared to TXN wild-type cells indicating, that TXN might regulate the transcriptional activation of Gadd45a by p53 in response to mild hyperthermia, although the involvement of p53 and Gadd45a in G_2/M arrest as a response to the other types of non-genotoxic stresses needed to be explored in future studies (Fig. 1A).

Recently, Gadd45a has been reported to bind to the cell cycle regulator Cdc2 and inhibit its kinase activity for induction of G_2/M arrest following genotoxic stress (40). Furthermore, some studies have suggested that Gadd45a might exclude the nuclear import of Cdc2 following UV treatment, although the localization of these interactions has not yet been clearly defined (25). Our data showed that TXN downregulation prevented the release of Cdc2 from the nucleus under mild hyperthermia, implying that TXN might affect Cdc2 localization with Gadd45a in order to induce G_2/M arrest in response to mild hyperthermia (Fig. 1B and C).

Furthermore, the cell cycle and mitotic index analyses showed a critical decrease in the fraction of cells in G_2/M phase in TXN siRNA-treated RKO cells as compared to TXN wild-type RKO cells (Fig. 2A and 3A). In addition, TXN siRNA-treated cells exhibited signs of apoptosis with a significant increase in the fraction of cells in the G_1 phase 36 h after heat treatment. These results were consistent with the cell viability data, although cytotoxicity was not induced by treatment with TXN siRNA (Fig. 2C), suggesting that endogenous TXN might affect the sensitivity of cells to heat stress in normal cells, although the effects of TXN inhibition in heat-treated cancer cells need to be investigated with regards to cancer therapy in future studies.

In conclusion, our study suggests that p53 activation in response to mild hyperthermia might be modulated by a TXN-dependent mechanism. Furthermore, we demonstrated for the first time the possible role of endogenously expressed TXN in mediating G_2/M arrest, accumulation of p53 and Gadd45a, and the export of Cdc2 to the cytosol induced upon mild hyperthermia. The detailed mechanism of TXN in mild hyperthermia-induced cellular responses might be explored in future studies. Understanding the distinct mechanisms involved in p53 activation and TXN function upon mild hyperthermia might assist in the use of non-genotoxic pathways to activate p53 for therapeutic or chemopreventive purposes.

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