

Research Paper

Inhibition of Heme Oxygenase-1 enhances hyperthermia-induced autophagy and antiviral effect

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Abstract

Hyperthermia has been clinically utilized as an adjuvant therapy in the treatment of cervical carcinoma. However, thermotolerance induced by heme oxygenase-1 (HO-1), a stress-inducible cytoprotective protein, limits the efficacy of hyperthermic therapy, for which the exact mechanism remains unknown. In the present study, we found that heat treatment induced HO-1 expression and decreased copy number of HPV16 in cervical cancer cells and tissues from cervical cancer and precursor lesions. Knockdown of HO-1 stimulated autophagy accompanied by downregulation of X-linked inhibitor of apoptosis protein. Furthermore, silencing of HO-1 led to cell intolerance to hyperthermia, as manifested by inhibition of cell viability and induction of autophagic apoptosis. Moreover, HO-1 modulated hyperthermia-induced, autophagy-dependent antiviral effect. Thus, the findings indicate that blockade of HO-1 enhances hyperthermia-induced autophagy, an event resulting in apoptosis of cervical cancer cells through an antiviral mechanism. These observations imply the potential clinical utility of hyperthermia in combination with HO-1 inhibition in the treatment of cervical cancer.

Key words: Heme oxygenase-1, autophagy, hyperthermia, cervical cancer, human papillomavirus

Introduction

Cervical cancer is the fourth most common cancer in women globally, and the leading cause of cancer related deaths in developing countries, where it is the second most common cancer in women [1, 2]. Persistent infection with certain types of human papillomavirus (HPV) causes nearly all cervical cancers [3]. The papillomavirus E2 proteins exert many functions in the viral cycle, including transcription regulation and DNA replication, which, in concert with modulating the host cells through direct protein interaction, play a pivotal role in carcinogenesis [4]. We and others have previously shown that moderate heat treatment (hyperthermia) induces HPV E2 gene mutation and promotes host

response *in vivo*, and induces apoptosis and cell cycle arrest of cervical cancer cells *in vitro* [5-8]. Hyperthermia has been applied clinically as a concomitant therapy for cervical carcinoma, and has been shown to improve the patient outcomes [7]. However, cervical carcinomas with the same histologic grade and HPV subtype may have significant differences in response to hyperthermia treatment [9]. Thus, further studies to identify potential molecular biomarkers for the effectiveness of thermal treatment are necessary.

On the other hand, heme oxygenase-1 (HO-1) is an inducible intracellular enzyme in response to oxidative stress, irradiation, ultraviolet light, hypoxia

and hyperthermia. Its catalytic action on heme produces three active products, namely, carbon monoxide, biliverdin and ferrous iron [10]. HO-1 itself and the by-products of heme degradation play a crucial role in maintaining cellular homeostasis, protecting cells from oxidative damage, attenuating inflammatory response and inhibiting cytokine production [11]. The cytoprotective function of HO-1 has been regarded as a shield from radiotherapy, chemotherapy, photodynamic therapy and other adjuvant therapy in several malignant diseases [12-14]. The function of anti-proliferation, anti-migration, and anti-invasion of HO-1 has also been reported in a number of cancer cell lines [15, 16]. Moreover, modulation of HO-1 has demonstrated therapeutic benefits by sensitizing cancer cells to therapies [13, 17, 18].

HPV produces oncoproteins to inhibit autophagy, thus suggesting a possible mechanism that autophagic dysfunction induced by HPV infection is an adaptive response to hinder viral clearance [19]. HO-1 can promote autophagy in the development of chemo-resistance in cells derived from leukemia, breast cancer, and renal cell carcinoma [20-22]. On the contrary, HO-1 may also inhibit autophagy to protect against cisplatin cytotoxicity in acute kidney injury [23]. Given the paradoxical effects of HO-1 on autophagy, we sought to investigate the expression of HO-1 in the hyperthermic condition and to explore the function and regulation of autophagy by HO-1 in cervical cancer in this study.

Materials and Methods

Patient specimen

This study was approved by the Ethical Committee of China Medical University (reference: [2017]2016-207-2). A total of 56 cervical biopsy specimens from June to October, 2017, were collected from the Department of Obstetrics and Gynecology, No.1 Hospital and Shengjing Hospital of China Medical University. Written informed consent was secured for all study participants.

The average age of the patients was 46.16 year. The specimens included 12 cervical intraepithelial neoplasia (CIN) I, 6 CINII, 4 CINIII, 26 cervical carcinomas and 8 patients without CIN (NCIN). Forty-two specimens harbored HPV16, 16 from CIN lesions and 26 from cervical carcinomas.

The fresh cervical tissue remnants after pathologic diagnosis (approximately 0.3 × 0.3 cm each) were equally splitted into two parts: one treated with 37 °C for 30 min, the other treated with 44°C for 30 min. Both parts were then submerged in culture medium at 37°C for 12 hours.

Cell culture

Human cervical cancer cell lines (HPV-negative C33A, HPV16 positive SiHa with 1-2 integrated HPV copies per cell, and HPV-positive CaSki containing 60-600 integrated HPV strands per cell [24]) were obtained from ATCC (Manassas, VA). Cells were maintained in RPMI-1640 medium (Biological Industries, CT, USA) with 10% fetal bovine serum (FBS, Biological Industries). All cell lines were incubated at 37°C in a humidified incubator containing 5% CO₂.

HPV16 integration and copy number of E6 analysis

HPV16 integration and copy number of E6 were analyzed by using real-time fluorescence PCR with type-specific primers and probes as described (Table 1) [25, 26]. The amplification conditions were 10 min at 95°C, followed by a two-step cycle of 95 °C for 15 seconds and 60 °C for 60 seconds, for 45 cycles. The total volume of 20 µl contained 20 pmol/µl of each primer, 10 pmol/µl of each probe, 10 µl volume of ready-to-use hot start PCR mix (FastStart Essential DNA Probes Master, Roche Diagnostics, Mannheim, Germany), and 100 ng of target DNA from biopsy specimens or cervical cancer cells. Two standard curves were obtained by amplification of a dilution series of 500 thousand to 500 copies of a clone of HPV16 in PML2d (Addgene, Teddington, UK). Ratios of E2 to E6 of less than 0.93 indicate the presence of two states of HPV16. Values of greater than 1 indicate the predominance of the episomal state.

Table 1. Primers and probes sequences of HPV16 E2 and E6

Name	Sense (5'→3')
HPV16E2F	ACCGAAGTATCCTCTCTGAAAATTATTAG
HPV16E2R	CCAAGGCGACGGCTTTG
HPV16E2PRO	VIC-CACCCCGCCGCGACCCATA-MGBNFQ
HPV16E6F	GAGAACTGCAATGTTTCAGGACC
HPV16E6R	TGTATAGTTGTTTCAGCTCTGTGC
HPV16E6PRO	6FAM-CAGGAGCGACCCAGAAAGTTACCACAGTT-MGBNFQ

F: forward primer; R: reverse primer; PRO: probe; MGBNFQ: quencher dye; 6-FAM: 6-carboxyfluorescein

Western Blot

Proteins were separated by electrophoresis on 12% sodium dodecyl sulfate polyacrylamide gel and electrophoretically transferred onto polyvinylidene difluoride membranes, which were then incubated with antibodies raised in rabbit against microtubule-associated protein 1A/1B-light chain 3 (LC3), HO-1, X-linked inhibitor of apoptosis protein (XIAP), p62, beclin1, ATG5, ATG7, mTOR, p-mTOR (Ser 2448), cleaved PARP (Cell Signaling Technology, Beverly, MA, USA) and GAPDH (Proteintech, Wuhan, China). Subsequently, the membranes were incubated

with horseradish peroxidase-conjugated goat anti-rabbit antibody (ZSGB-BIO, Beijing, China) and the protein bands were examined using ECL Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA).

Flow cytometry

Cellular apoptosis was detected by using APC (eBioscience, San Diego, CA, USA) or FITC Annexin-V Apoptosis Detection Kit (BD Biosciences, USA) in accordance with the manufacturer's instructions. The adherent cells were trypsinized and washed with cold Phosphate Buffered Saline (PBS). Cell suspension was incubated with 5 μ l of APC or FITC Annexin-V, and 5 μ l of propidium iodide (PI) for 15 min in the dark at room temperature. For cell cycle analysis, the cells were fixed with 75% cold ethanol overnight, and each sample was incubated with 5 μ l of PI (40 μ g/ml) and 5 μ l of RNase A (10 μ g/ml) at 37 °C for 20 min. After staining, samples for apoptosis and cell cycle distribution were analyzed using BD LSRFortessa (BD Biosciences) and the percentage of cells at sub-G1, G1, S, and G2/M phase was analysed using ModFit software (Becton Dickinson, USA).

RNA isolation and real-time PCR

Total RNA was isolated with miRNeasy mini kit (Qiagen, Stockach, Germany) and then converted to cDNA by using GoScript™ Reverse Transcription System (Promega, Madison, WI). Real-time PCR was performed to detect relative mRNA expression level using primers as described in Table 2.

Table 2. Primer sequences for the mRNA quantification by real-time PCR

Name	Sense (5'→3')	Antisense (5'→3')
HO-1	GTCAGGCAGAGGGTGATAGAAG	GTGTAAGGACCCATCGGAGAAG
TP53	GATGTTCCGAGAGCTGAATGAG	TATGGCGGGAGGTAGACTGA
OAS1	ACTACCAGCAACTCTGCATCTA	CTCCACCACCCAAGTTTCCT
MX1	CCTCGTGTCCACCTGAAGA	CTCCAGCAGATCCCTGAAATATG
AP1S1	TGTGCGAGCTGGACATCAT	CATCCTCTCTGTCAGTAGGT
MYD88	TAAGAAGGACCAGCAGAGCC	CATGTAGTCCAGCAACAGCC
AIP5	CGACCTAGAACAGACCTTCAAT	GTTAGGGAGAACCCTGCTCAC
CASP3	ACAGACAGTGGTGTGATGATG	GCACAAAGCGACTGGATGAA
CytC	ACTCTTACACAGCCGCAAT	CTTCCTTCTCTTAATGCCGAC
GAPDH	AAGAGCACAAGAGGAAGAGAG	GTCTACATGGCAACTGTGAG
	AGAC	GAG

RNA interference

HO-1 or XIAP validated small interfering RNA (siRNA) as well as scrambled siRNA were purchased from Life Technologies Corporation (Foster City, CA). The siRNA sequences are listed in Table S1. Cells were transfected with Silencer® Select Validated HO-1 siRNA or XIAP siRNA, and mixed with

Lipofectamine™ RNAiMAX (Thermo Fisher scientific) as transfection reagent according to the manufacturer's protocol.

Cell viability assay

The cells were seeded in 96-well plates at a density of 4000 cells per well, following the treatment of the cells with 3-Methyladenine (3-MA)/Rapamycin (Sigma, St. Louis, MO, USA) for 2 hours or HO-1 siRNA for 24 hours. The cells were then treated with hyperthermia at 44°C for 30 min. At day 0, 1, 2 and 3, 20 μ l of MTS solution (Promega) was added to the medium and the cells were incubated at 37°C for 3 hours. The absorbance was measured at 490nm using a spectrophotometer.

Tandem mass tag (TMT) labeling and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

CaSki cells were transfected with HO-1 siRNA or scrambled siRNA. At 48 hours, protein was extracted 48 hours after transfection by using lysis buffer with 1% protease inhibitor cocktail (Sigma), and reduced with 5 mM dithiothreitol for 30 min at 56°C and 11 mM iodoacetamide for 15 min at room temperature in darkness. Peptides were labeled using TMT kit and then separated by high-performance liquid chromatography (HPLC), and combined into 18 fractions. The peptides were then subjected to NSI source followed by tandem mass spectrometry in Orbitrap Fusion™ Lumos™ Tribrid™ (Thermo) coupled online to the Ultra Performance Liquid Chromatography (UPLC). The resulting data were processed using Maxquant search engine (v.1.5.2.8). Each experiment was repeated three times.

Statistical analysis

All experiments were repeated at least three times and quantitative values were expressed as mean \pm SD. Statistical significance was determined by one-way analysis of variance (ANOVA) or paired or unpaired *t*-test for comparisons of group means. A *p*<0.05 was considered statistically significant.

Results

Hyperthermia induces HO-1 expression in cervical cancer cells and tissues from cervical cancer and its precursors

We first examined the effect of hyperthermia on a number of key genes influencing cell fate in 3 human cervical carcinoma lines. Cells were treated with 37 °C and 44 °C by water baths for 30 minutes, respectively. As shown in Figure 1A, there was a sustained increase in HO-1 mRNA expression 6 hours after heat-treatment at 44°C, with C33A being the

most thermo-sensitive line that showed a near 30-fold increase. There was only an approximately 2-5 fold increase in HO-1 mRNA expression in CaSki and SiHa cells (Figure S1). In contrast, there were no significant changes in the expression of the other genes examined. In keeping with this finding, western blot analysis showed hyperthermia-induced HO-1 protein expression in a time-dependent manner, peaked at 12-24 hours (Figure 1B).

To further validate these observations *ex vivo*, we expanded our investigation into cervical biopsies. Forty-seven fresh samples from 21 patients with and without cervical cancer precursor lesions [7 CIN I, 5 CIN II, 4 CIN III, and 5 NCIN] and 26 patients with cervical cancer were equally splitted and treated with 37°C and 44°C, respectively, by water bath for 30 minutes. In concert with the findings in cervical cancer cell lines, hyperthermia induced an average of 3.5- and 2.9-fold increase in HO-1 mRNA expression in cervical cancer precursor and cervical cancer tissues, respectively (Figure 1C). In regard to different grades of CIN lesions, hyperthermia drastically

induced HO-1 mRNA expression of in CIN I, with an average of 7 fold, while no significant changes were found in HO-1 expression in CINs II and III (Figure 1D).

Identification of dysregulated proteins in HO-1 silenced cervical cancer cells

To explore the dysregulated proteins regulated by HO-1 in cervical cancer cells, tandem mass tag (TMT) labelling and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis were performed to identify the differentially expressed proteins between the HO-1 siRNA and scrambled siRNA expressing CaSki cells. To that end, a total of 6918 molecules were identified. Based on the criteria of $p < 0.05$ and fold change ≥ 1.2 , 191 upregulated and 496 downregulated proteins were displayed in HO-1 silenced cells (Figure 2A). It was of substantial interest that a number of cell cycle-, apoptosis- and phagocytosis-related molecules were identified among these 687 differentially expressed proteins, including XIAP, caspase activity and apoptosis

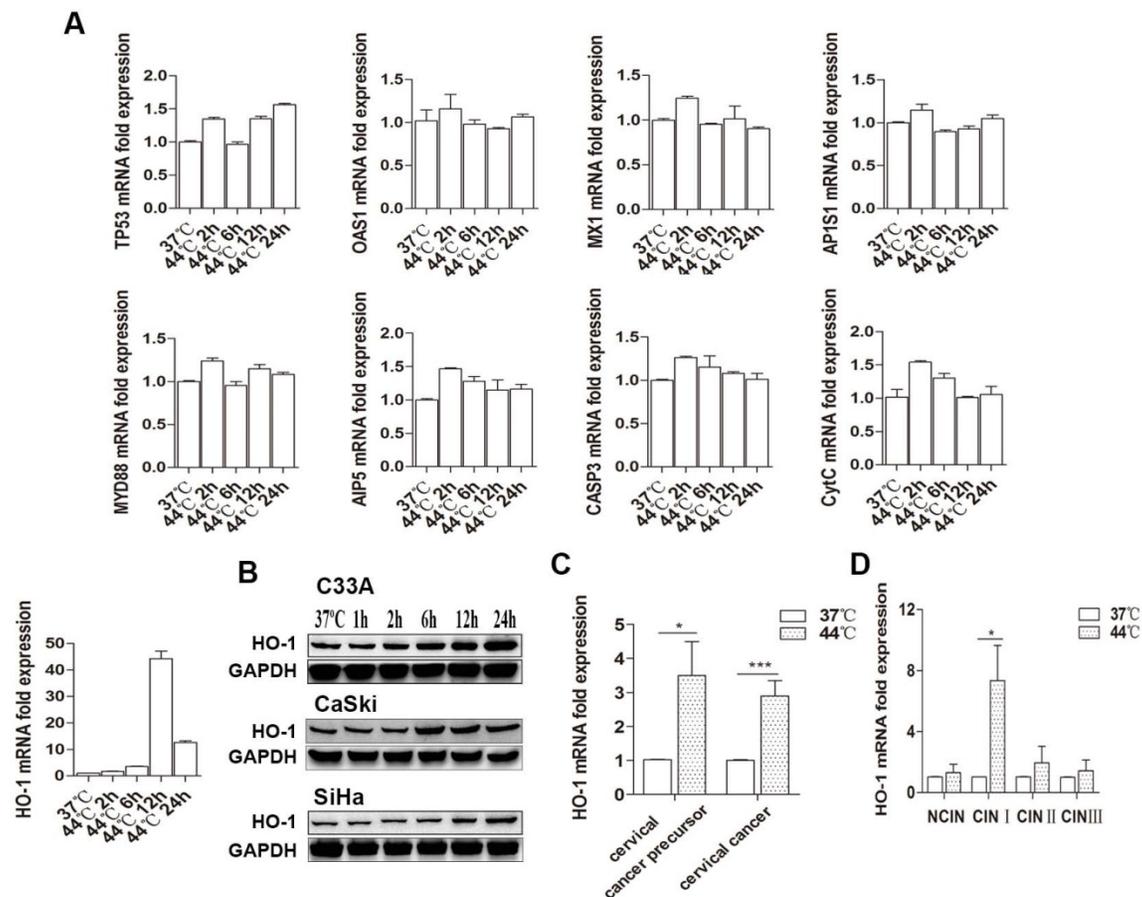


Figure 1. Hyperthermia induces HO-1 expression in cervical cancer cells and tissue. (A) Hyperthermia induces HO-1 mRNA expression in a time-dependent manner in C33A cells, but does not alter the expression of other genes examined. **(B)** Hyperthermia induces HO-1 protein expression in a time-dependent manner in all three cervical cancer cell lines. **(C)** Hyperthermia induces HO-1 mRNA expression in cervical cancer and precursor tissues *ex vivo*. **(D)** Hyperthermia significantly induces HO-1 mRNA expression in CIN I lesions *ex vivo*. All data were normalized against the endogenous control of GAPDH (* $p < 0.05$, *** $p < 0.001$, by paired t-test). Abbreviations: HO-1, Heme oxygenase-1; TP53, cellular tumor antigen p53; OAS1, 2'-5'-oligoadenylate synthase I; MX1, myxovirus resistant I; AP1S1, AP-1 complex subunit sigma-1A; MYD88: myeloid differentiation factor 88; AIP5, atrophin-interacting protein 5; CASP, caspase; CytC, cytochrome C.

inhibitor 1 (CAAP1), programmed cell death protein 6 (PDCD6), cyclin-dependent kinase inhibitor 1A (CDKN1A), cyclin-dependent kinase 6 (CDK6), and LC3 (Figure 2B).

Inhibition of HO-1 promotes hyperthermia-induced autophagy

HO-1 may act as either a positive regulator of the cytoprotective autophagy or a negative mediator to inhibit cytotoxic autophagy in different therapeutic chemical- or physical-induced responses [22, 23]. Western blot analysis showed that both knockdown of HO-1 and hyperthermia at 44 °C promoted the

conversion of LC3-I (cytosolic form) to LC3-II (membrane-bound), a process to initiate formation and lengthening of the autophagosome, currently considering as a simple, quick procedure to verify the presence of autophagy [27]. Meanwhile, either silencing HO-1 or hyperthermia reduced the expression of p62 and p-mTOR. Furthermore, there was a further increase in the LC3-II/LC3-I ratio and a further downregulation of p62 and p-mTOR with both hyperthermia and HO-1 silencing, suggesting a superior therapeutic effect with the combined strategy over the single agent-induced autophagy (Figure 2C).

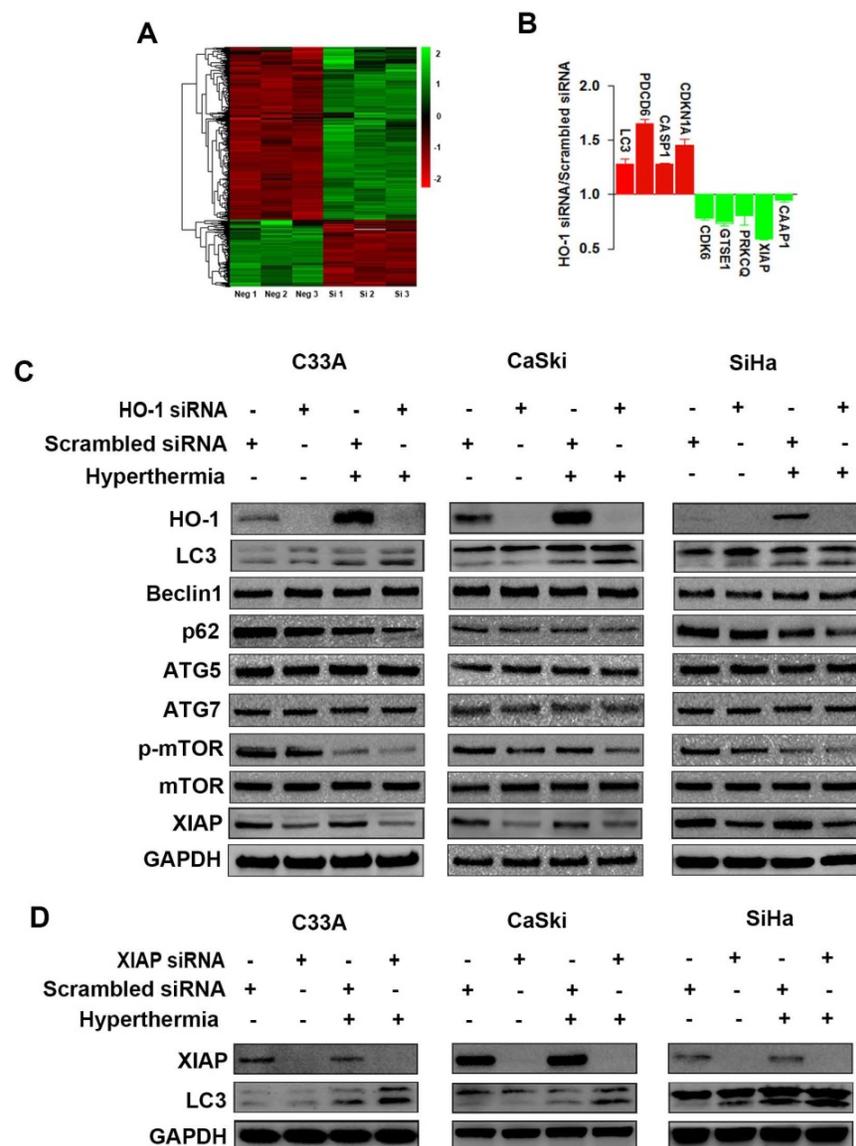


Figure 2. Hyperthermia and HO-1 regulate key molecules in autophagy. (A) Differentially expressed proteins between HO-1 siRNA and scrambled siRNA CaSki cells by LC-MS/MS analysis. (B) Illustration of differentially expressed cycle-, apoptosis- and phagocytosis-related molecules. (C) Hyperthermia enhances downregulation of XIAP, p62, and p-mTOR and upregulation of LC3 induced by HO-1 silencing in three cervical cancer cell lines. The cells were transfected with HO-1 siRNA or scrambled siRNA for 24 hours prior to exposure to hyperthermia, and the proteins were analyzed by western blot. (D) Hyperthermia enhances conversion of LC3-I to LC3-II induced by XIAP silencing. The cells were transfected with XIAP siRNA or scrambled siRNA for 24 hours prior to exposure to hyperthermia, and the proteins were analyzed by western blot. Abbreviations: LC3, microtubule-associated protein 1 light chain 3; XIAP, X-linked inhibitor of apoptosis; PDCD6, programmed cell death protein 6; CDKN1A, cyclin-dependent kinase inhibitor 1; CDK6, cyclin-dependent kinase 6; GTSE1, G2 and S phase-expressed protein 1; PRKCO, protein kinase C theta type; CAAP1, caspase activity and apoptosis inhibitor 1; CASP, caspase.

XIAP, an anti-apoptotic molecule, has been recently recognized as a negative regulator of autophagy [28]. Our research to that end revealed that silencing of HO-1 inhibited the expression of XIAP. Thus, we next sought to examine whether XIAP plays a role in regulating autophagy. As shown in Figure 2D, knockdown of XIAP by siRNA led to increased conversion of LC3-I to LC3-II in three cervical cancer cell lines, an event further enhanced by hyperthermia. These findings indicate that HO-1 inhibits autophagy via downregulation of XIAP.

Knockdown of HO-1 inhibits the viability of cervical cancer cells

To examine whether hyperthermia and HO-1 affect the viability of cervical cancer cells, MTS cell proliferation assay was carried out. Compared to the control group, the cell viability was reduced two days after heat treatment or HO-1 knockdown. The combination of hyperthermia and HO-1 silencing further diminished the cell viability, suggesting that inhibition of HO-1 facilitates heat-mediated down-regulation of cervical cancer cell proliferation (Figure 3A, Figure S2A). In a parallel study, blockage of autophagy induction by 3-MA increased the cell viability, while induction of autophagy by rapamycin significantly sensitized cervical cancer cells to heat-induced cytotoxicity (Figure 3B, Figure S2B). These data indicate that upregulation of autophagy by HO-1 knockdown and hyperthermia provides a synergistic effect in inhibiting cervical cancer cell viability.

Absence of HO-1 augments hyperthermia-induced autophagic apoptosis

We have previously shown that local hyperthermia induces apoptosis of keratinocytes infected with HPV and melanoma cells [8,29]. Apoptotic cells are characterized by DNA fragmentation, among other typical features, that can be analysed by PI flow cytometric assay [30]. Apoptosis can be determined by quantifying the sub-G1 peak [31]. To explore the potential effect of HO-1 knockdown and hyperthermia on apoptosis of cervical cancer cells, the sub-G1 assay was first carried out. After transfection with either HO-1 siRNA or scrambled siRNA, cells were subjected with hyperthermia at 44°C for 30 minutes. While the sub-G1 peak was negligible in the control cells (0.48% in C33A), there was a 1.33-fold increase in the cells treated with HO-1 siRNA, and a 1.92-fold increase in the cells treated with hyperthermia. The proportion of sub-G1 peak further increased to an average of 6.7-fold in the cells treated with both HO-1 siRNA and hyperthermia ($p < 0.01$; Figure 3C). The data obtained

using CaSki and SiHa cells were shown in Figure S2C.

Apart from the sub-G1 assay, the total number of apoptotic cells in each condition was further quantitated by flow cytometry analysis. To that end, there were a 2.04-fold increase with hyperthermia, a 1.52-fold increase with HO-1 siRNA, and a 2.97-fold increase with their combination in C33A cells ($p < 0.001$; Figure 3D). Similar observations were obtained when using CaSki and SiHa cells (Figure S2D).

Autophagy is involved in the process of various forms of regulated or unregulated cell death [32]. We next asked if autophagy plays a role in the apoptosis of cervical cancer cells induced by hyperthermia and HO-1 silencing. To that end, 3-MA attenuated heat-induced apoptosis and the expression of cleaved PARP, whereas rapamycin, an autophagy agonist, increased the proportion of apoptotic cells and upregulated cleaved PARP. Furthermore, 3-MA decreased HO-1 knockdown-induced apoptosis and cleaved PARP expression, with or without hyperthermia (Figure 3E and F). Likewise, similar observations were obtained in CaSki and SiHa cells (Figure S2E and S2F). Thus, the collective findings indicate that autophagy is involved in heat-induced apoptosis.

In order to further clarify the crosstalk between apoptosis and autophagy, our data revealed that zVAD-fmk, an inhibitor of pan-caspase, led to increased conversion of LC3-I to LC3-II in three cervical cancer cell lines, with or without HO-1 silencing (Figure 3G and S3G).

Hyperthermia reduces the copy number of HPV16 E6 in cervical biopsy specimens

In addition to viral load, a number of other HPV-related factors are also thought to predispose cervical cancer, of which viral integration status is a key parameter and has been widely investigated [25, 33]. We thus examined HPV16 copy numbers and integration status over hyperthermia treatment in the CIN and cervical cancer tissue samples. The average viral loads from different grades of CIN lesions varied greatly (ranged 1.252×10^3 to 1.3825×10^7 copies per 50 ng DNA), and the copy number declined after hyperthermia treatment (1.102×10^3 to 4.2586×10^6 copies), especially in CIN I and CIN II, although it did not reach a statistical significance (Figure 4A and 4B). The average viral loads in cervical cancer samples decreased significantly, from 2.35×10^7 to 1.51×10^7 copies, with an average reduction of 35.7% ($p < 0.05$; Fig. 4C). Five samples (1 CIN I and 4 cervical cancer) with mixed forms (integrated and episomal) of HPV changed to pure episomal form, where equivalent amounts of E2 and E6 were detected. Four cervical

cancer samples harbored integrated form of HPV, where the E2/E6 ratios were low (0.1) [25], either before or after hyperthermia treatment. Four cervical cancer samples altered from purely integrated form to mixed form after treatment. In the 14 cancer samples harbouring mixed forms of HPV16, the integration degree increased in 2, and significantly reduced in 12 (average E2/E6 ranging 39.38% to 60.1%) after hyperthermia treatment. While no alteration in the HPV16 copy number was observed in CIN lesions with hyperthermia, the integration degree was markedly decreased (average E2/E6 ranging 31.58% to 57.23%) (Figure 4D-4F).

HO-1 modulates autophagy-induced HPV16 E6 copy number alteration

Recent studies have demonstrated that inhibition of autophagy results in enhancement of HPV infectivity and prevention of viral clearance [34, 35]. These findings prompted us to investigate if altered autophagy affects the HPV infectivity in cervical cancer cells. As illustrated in Figure 4G, pharmacologic induction of autophagy by rapamycin significantly decreased, and inhibition of autophagy by 3-MA significantly increased the HPV16 copy numbers, respectively.

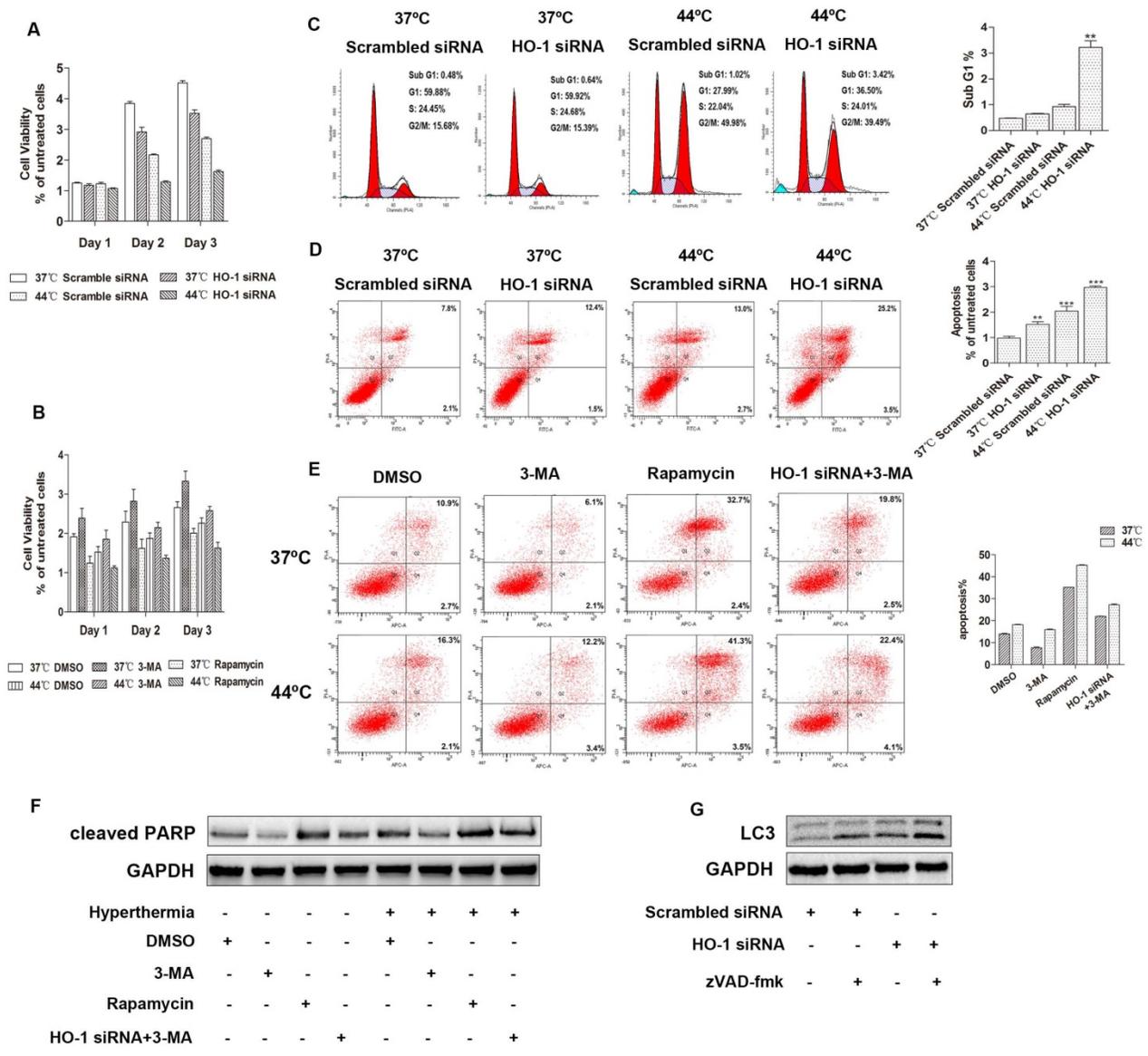


Figure 3. Downregulation of HO-1 promotes autophagic apoptosis in cervical cancer cells. (A) HO-1 knockdown facilitates hyperthermia-mediated down-regulation of cervical cancer cell proliferation. C33A cells transiently transfected with scramble and HO-1 siRNA were incubated for 24 hours at 37°C or 44°C, cell viability was measured by MTS assay on day 1, 2 and 3. (B) Autophagy inhibitor 3-Methyladenine (3-MA) attenuates cervical cancer cells to hyperthermia-induced cytotoxicity, and autophagy inducer rapamycin sensitizes cervical cancer cells to hyperthermia-induced cytotoxicity. C33A cells were pretreated with DMSO, 3-MA (10 mM), or rapamycin (100nM) for 2 hours and then incubated at 37°C or 44°C prior to MTS assay. (C) Comparison of the sub G1 phases in C33A cells treated with hyperthermia and/or HO-1 siRNA by flow cytometry analysis. (D) Flow cytometric analysis of apoptosis of C33A cells treated with hyperthermia and/or HO-1 siRNA. (E) Flow cytometric analysis of apoptosis of C33A cells treated with 3-MA, rapamycin, or HO siRNA, with or without hyperthermia. Cells were incubated with DMSO, 3-MA (10 mM), or rapamycin (100 nM), or transfected with HO-1 siRNA 24 hour prior to exposure to 3-MA, for 2 hours before treated with hyperthermia. Cleaved PARP was detected by western blot (F). (G) C33A cells transfected with HO-1 siRNA alone or in combination with zVAD-fmk (20 μM) for 24 h, and the proteins were analyzed by western blot. The experiment was replicated three times. **p<0.01, ***p<0.001 compared to the control group (one way ANOVA). Each point represents the mean ± SD.

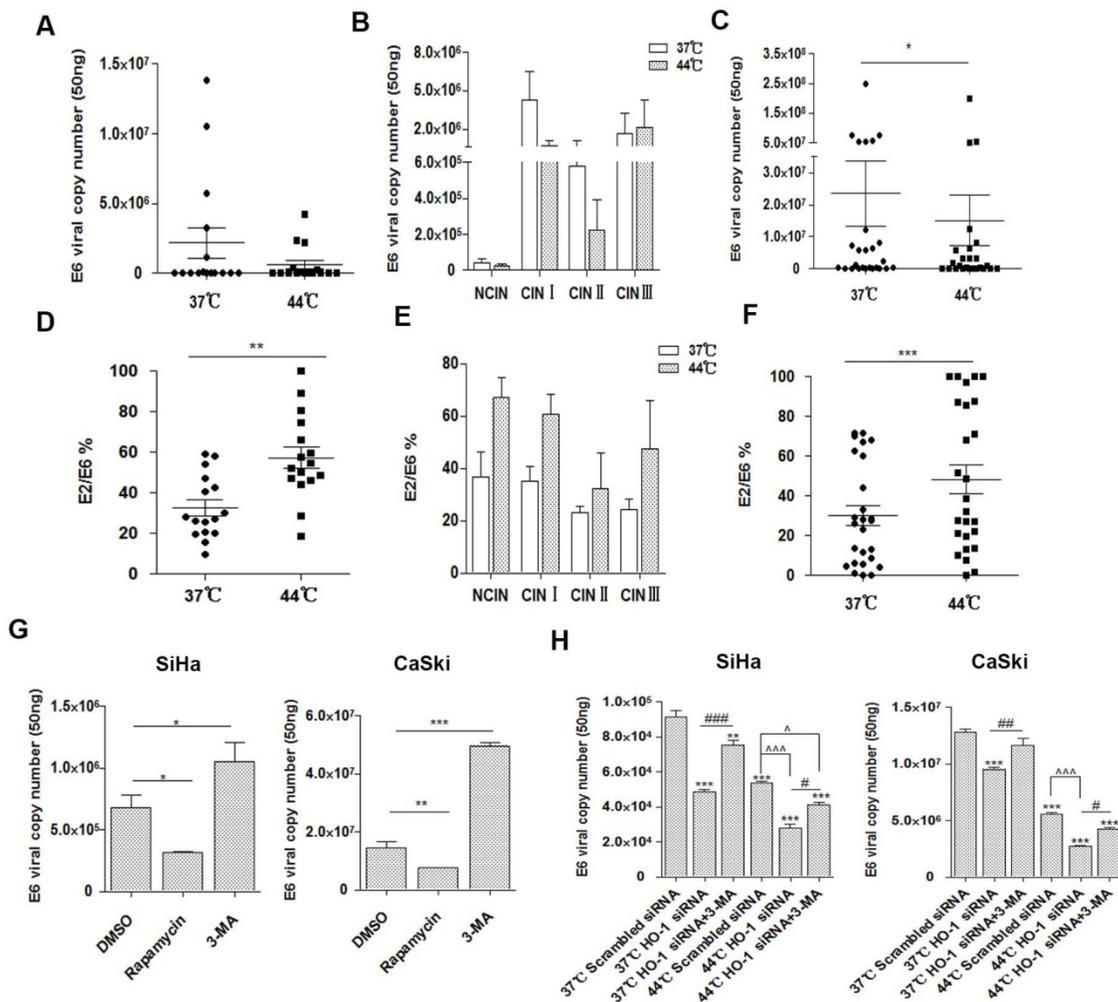


Figure 4. Inhibition of HO-1 enhances hyperthermia-induced, autophagy-dependent antiviral effect. (A) Hyperthermia decreases the total number of HPV16 E6 (episomal and integrated) copies in NCIN-CIN III lesions. Horizontal bars represent means. (B) Hyperthermia modestly decreases HPV16 E6 copy numbers in CIN I and CIN II lesions. (C) Hyperthermia significantly decreases HPV16 E6 copy numbers in cervical cancer tissue. (D) Hyperthermia significantly alters HPV16 integration in CIN lesions, although no statistical significance is reached when compared to the individual grade. (E) (F) Hyperthermia significantly affects HPV16 integration in cervical cancer samples. (G) Pharmacologic induction of autophagy by rapamycin significantly decreases, and inhibition of autophagy by 3-MA significantly increases the HPV16 copy numbers in cervical cancer cells. SiHa cells and CaSki cells were pretreated with DMSO, 3-MA (10 mM), or rapamycin (100 nM) for 12 hours and DNA was extracted to analyze the copy numbers of HPV16 E6. (H) Hyperthermia and HO-1 knockdown significantly decreased HPV16 E6 copy numbers in cervical cancer cells. HPV16 E6 copy numbers were analyzed in SiHa cells and CaSki cells treated with HO-1 siRNA transfection, hyperthermia, or their combination, in the presence or absence of 3-MA. **p*<0.05, ***p*<0.01, ****p*<0.001 (paired t-test in A-F, one way ANOVA in G and H). # and ^ *p*<0.05, ###*p*<0.01, #### and ^^^ *p*<0.001 (unpaired t-test).

The role of HO-1 in antiviral activity remains a matter of debate. Thus, we next examined if HO-1 was involved in the modulation of HPV16 E6 copy number in cervical cancer cells. As shown in Figure 4H, both hyperthermia and HO-1 knockdown resulted in markedly decreased HPV16 E6 copy numbers in SiHa cells (41.3% and 46.8%, respectively) and CaSki cells (56.6% and 25.7%, respectively). The combination of hyperthermia and HO-1 silencing reduced the viral load by 69.3% in SiHa cells and 78.9% in CaSki cells, respectively, when compared to the control group.

To further verify whether HO-1 modulates autophagy-induced antiviral effect, we examined the viral copy numbers in the HO-1 knockdown group treated with 3-MA simultaneously. To that end, inhibition of autophagy by 3-MA significantly

dampened the HO-1 silencing-induced viral load reduction (Figure 4H). Thus, upregulation of autophagy by HO-1 knockdown effectively reduces HPV16 E6 viral load in cervical cancer cells.

Discussion

In this study, we demonstrated that HO-1 was overexpressed in HPV infected cells and tissue subjected to hyperthermia, especially in low grade CIN lesions. The reason for this result is probably that the small sample size limits to some extent the generalization of the finding or that low grade CIN lesions are more sensitive to hyperthermia, so further study is needed in the future. While HO-1 is considered as the first line of cellular defense of cancer cells against the insults, the exact mechanism underlying this protective effect remains unclear. The

present study explored the function and regulatory mechanism of autophagy by HO-1, in hyperthermia-stimulated conditions.

To discern the regulatory molecules of HO-1 in cervical cancer cells, we first carried out proteomic analysis to identify differentially expressed proteins. The research to that end has identified a number of cell cycle and apoptosis-related molecules among the 687 abnormally expressed proteins, including XIAP, CAAP1, CDK6, PDCD6, CASP1 and CDKN1A. Furthermore, upregulation of LC3 indicated a potential role of LC3-associated phagocytosis in this event. Inhibition of phagocytosis reportedly leads to severe virus infection and early death, which may partly explain the observation of viral load reduction induced by HO-1 knockdown in our study [35-38]. There is considerable evidence that HO-1 induction may provide a potential therapeutic effect by stimulating an innate cellular response, decreasing viral replication and inflammation against a variety of virus, such as Ebola virus, human immunodeficiency virus, hepatitis B and C virus, enterovirus 71, influenza virus, dengue virus and respiratory syncytial virus [39-41]. Similarly, inhibition of HO-1 provides a promising therapeutic strategy against human herpesvirus-8-related malignancies [42]. The controversial topic of the effect of HO-1 on antiviral ability remains in dispute. Our findings indicate that silencing HO-1 reduces viral copy number, a process likely autophagy-dependent.

Previous studies have suggested the disruption status of viral E2 gene concomitant with viral load as a potential mechanism for HPV16 positive cervical cancer pathogenesis [43]. Concurrent hyperthermia and chemoradiation has been utilized clinically given its effects in disrupting cell cycle, inhibiting cell proliferation, inducing apoptosis and mutating HPV [5-7, 44]. In the present study, we found that hyperthermia not only reduced the copy number, but also changed the physical state of HPV16, decreased the degree of viral integration in most cervical cancer and precursor lesions. Meanwhile, knockdown of HO-1 increased heat-induced apoptosis and inhibited cell viability. Inactivation of HO-1 also exacerbated the autophagic response and decreased viral copy number. These findings, along with previous observations, suggest that inhibition of HO-1 is an effective modality in sensitizing cancer cells to therapies [13, 17].

Autophagic and apoptotic signalling pathways almost always intertwine to affect cell fate [45]. The research to that end has resulted in conflicting observations. Some authorities consider that autophagy augments pro-apoptotic function of chemotherapeutic agents, while others believe that

autophagy may enhance survival of tumor cells [46-48]. We found that autophagy promoted heat-stimulated autophagic apoptosis in cervical cancer cells. An early study has demonstrated that in addition to accelerating cell death induced by hyperthermia, autophagy can prevent virus duplication and infection in accordance with our finding that autophagy induced by inhibition of HO-1 decreases virus load in cervical cancer cells [49].

In summary, the data in the present study suggest a possible biologic mechanism for thermosensitization of cervical cancer cells by silencing of stress-inducible enzyme, HO-1. These findings provide evidence that patients with cervical cancer may benefit from concurrent HO-1 silencing and hyperthermia treatment given their additive effect in activating autophagic apoptosis and antiviral properties. Whether targeting downstream molecules of HO-1 may overcome hyperthermia resistance is worth further exploring. Moreover, HO-1 has been recognized to have major immunomodulatory and anti-inflammatory properties, while hyperthermia may induce antiviral activity by endogenous interferon-dependent pathway [50]. Thus, other potential mechanisms by which inhibition of HO-1 promotes hyperthermia-induced viral clearance in cervical cancer cells remain to be investigated.

Abbreviations

HPV: human papillomavirus; HO-1: Heme oxygenase-1; LC3: microtubule-associated protein 1 light chain 3; XIAP: X-linked inhibitor of apoptosis; TMT: Tandem mass tag; PI: propidium iodide; HIV: Human Immunodeficiency Virus; KSHV: Kaposi's Sarcoma-associated Herpesvirus; IFN: interferon; siRNA: small interfering RNA; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; MTS: 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium; TP53: cellular tumor antigen p53; OAS1: 2'-5'-oligoadenylate synthase 1; MX1: myxovirus resistant 1; AP1S1: AP-1 complex subunit sigma-1A; MYD88: myeloid differentiation factor 88; AIP5: atrophin-interacting protein 5; CASP: caspase; CytC: cytochrome C; PDCD6: programmed cell death protein 6; CDKN1A: cyclin-dependent kinase inhibitor 1; CDK6: cyclin-dependent kinase 6; GTSE1: G2 and S phase-expressed protein 1; PRKCC: protein kinase C theta type; CAAP1: caspase activity and apoptosis inhibitor 1; PBS: Phosphate Buffered Saline.

Supplementary Material

Supplementary figures and table.

<http://www.ijbs.com/v15p0568s1.pdf>

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Author contributions

Y.Y. H.-X. W. and L.Z. performed biochemical experiments, and participated in data analysis. W.H., X.-D. L. and R.-Q. Q. conceived the idea, participated in the design of the study and interpretation of data. X.-Y. S., W.S. and X.-H. G. participated in the intellectual input and supervision. S.H. and L.C. drafted the manuscript with contributions from other authors.

Competing Interests

The authors have declared that no competing interest exists.

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