Abstract. Heme oxygenase (HO)-1 is a heat shock protein induced by hyperthermia, responsible for cellular resistance to temperature. The aim of this in vitro study was to clarify the response of gastric and ovarian cancer cells to hyperthermic intraperitoneal chemotherapy, following the modulation of HO-1 expression. AGS and OVCAR-3 cells were treated with different temperature regimens, either alone or in combination with an IC₅₀ dose of cisplatin for 1 h. Prior to treatment, HO-1 expression was silenced by short interfering RNA transfection. In OVCAR-3 cells, cisplatin increased HO-1 mRNA expression by 3.73-fold under normothermia and 2.4-fold under hyperthermia; furthermore, these factors similarly increased HO-1 protein expression levels. Exposure to cisplatin under hyperthermia reduced the viability of OVCAR-3 cells by 36% and HO-1-silencing enhanced this effect by 20%. HO-1-silencing under normothermia increased apoptotic rates in cisplatin-treated OVCAR-3 cells by 2.07-fold, and hyperthermia enhanced the effect by 3.09-fold. Semi-quantitative polymerase chain reaction (PCR) cell analysis indicated that exposure to cisplatin decreased the cell index under normothermia, and that hyperthermia boosted this effect in OVCAR-3. In AGS cells, only temperature increased cellular HO-1 levels. Silencing HO-1 in AGS cells at 37°C reduced viability by 16% and increased apoptotic rates 2.63-fold. Hyperthermia did not affect AGS viability, however, apoptosis was increased 6.84-fold. PCR analysis indicated no additional effects of hyperthermia on the AGS cell index. HO-1 is induced in cancer cells by different stressors in a variable manner. In tumors with highly inducible HO-1, prior silencing of this gene could improve the cellular response to hyperthermia and cisplatin.

Introduction

The response and resistance of cancer cells to chemotherapy and hyperthermia depend on the induction and expression of a number of cytoprotective proteins, including Hsp70 and Hsp27 (17-19). Therefore, the modulation of cytoprotective proteins may serve a crucial role in cancer treatment. One potential target is heme oxygenase (HO)-1, particularly in HIPEC, since high temperatures are a component of HIPEC, and it has been reported that, under hyperthermia, cells enhance HO-1 expression for self-protective purposes (19). HO-1 is normally expressed at low levels in the majority of tissues, including the gastrointestinal tract, female reproductive organs, brain and bone marrow (20); however, it is highly inducible by a variety of stimuli, including cytokines, lipopolysaccharides (21) and...
serine/threonine kinases (22). Cellular levels of HO-1 are known to be temperature-dependent (23,24). HO-1 is overexpressed under hyperthermic conditions, exerting a protective function (25,26).

An in vitro study was conducted to clarify the underlying mechanism of how cisplatin and hyperthermia induce HO-1 expression in ovarian and gastric cancer cells. In addition, the present study investigated the response of these cancer cells to cisplatin and hyperthermia following the modulation of HO-1 protein expression.

Materials and methods

Cell lines and conditions. Human gastric adenocarcinoma, AGS, and ovarian adenocarcinoma, OVCAR-3, cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). OVCAR-3 cells were cultivated in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 20% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 1% penicillin/streptomycin and 0.01 mg/ml bovine insulin (Gibco; Thermo Fisher Scientific, Inc.). AGS cells were harvested in Ham's F-12K medium with 10% FBS and 1% penicillin/streptomycin at 37˚C and 5% CO₂.

Experimental design. Cells were harvested for 24 h in the conditions described previously. The cells were subjected to conditions of normothermia (37˚C) or 43˚C and/or cisplatin exposure lasted for 1 h; this step began once the medium reached the desired temperature (37  or 43˚C), as measured by a digital thermometer in a humid incubator with a set temperature of 43˚C. Following treatment, the medium was changed and cells were harvested after 48 h of incubation using Annexin V-PE and 7-aminoactinomycin D. A Guava Analysis Flow Cytometer (Guava; EMD Millipore, Billerica, MA, USA) was used to determine cell viability, apoptosis, and cell index were all subsequently measured. Additionally, these cell lines were used in real time cell analysis, western blotting and semi-quantitative polymerase chain reaction (qPCR) assays.

Silencing of HO-1. HO-1 small interfering RNA (siRNA; 30 nM HMOX1; sense 5'-UUCUCCGAACGUGUCACGU-3' and antisense 5'-GUCAUCCAGGAGAATGGC-3') was obtained from Ambion; Thermo Fisher Scientific, Inc., and negative control (30 nM AllStars Negative Control siRNA; sense 5'-UUCCGGAACGUGUCAG-3' and antisense 5'-ACGUGACACGUUCCGAGA-3') was obtained from Qiagen GmbH (Hilden, Germany). Lipofectamine® RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.) and Opti-MEM™ media (Gibco; Thermo Fisher Scientific, Inc.) were used according to the manufacturer's protocols. The efficiency of transfection was verified using BLOCK-it Alexa Fluor (Invitrogen; Thermo Fisher Scientific, Inc.). The efficiency of knockdown was verified by western blot analysis. HO-1-silencing was performed 72 h prior to implementation of the experimental temperature and treatment with cisplatin.

MTT assay. Cell viability was determined using an MTT assay (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were incubated for 4 h at 37˚C following the addition of 5 mg/ml MTT reagent. The supernatant was subsequently removed and dimethyl sulfoxide was added (Carl Roth GmbH Co KG, Karlsruhe, Germany). Absorbance was measured at a wavelength of 570 nm and the reference was measured at 650 nm using a Sunrise spectrophotometer (Tecan Austria GmbH, Grödig, Austria).

qPCR. Cellular RNA was extracted using a PureLink® RNA Mini kit (Ambion; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. Purified RNA was measured and verified for purity using ultraviolet (UV) spectrophotometry (Nanodrop; Thermo Fisher Scientific, Inc.). Using the Super Script Vilo Master Mix (Invitrogen; Thermo Fisher Scientific, Inc.) with 2 µg RNA, cDNA was generated, according to the manufacturer's protocols. RNA amplification was performed in a 20 µl reaction volume, which contained 1X PCR Master Mix, primers, and 2 µl cDNA template. Thermocycling conditions were as follows: initial step at 95˚C for 10 min (1 cycle), denaturation at 95˚C for 15 sec and annealing/extension at 60˚C for 1 min (40 cycles), followed by a final extension step at 72˚C for 2 min. HO-1 primers were obtained from Invitrogen (Thermo Fisher Scientific, Inc.): forward, 5'-TGCTCAACATCCAGCTTTTGAGA-3'; and reverse, 5'-CAGGCAGAGAATGCTGAGTTC-3'. The products were loaded on 1.5% agarose gels. Ethidium bromide staining and UV light (Gel Doc™ XR+ Gel Documentation System; Bio-Rad Laboratories, Inc., Hercules, CA, USA) were used for visualization. Analysis was performed using ImageLab software (version 6.0.0; Bio-Rad Laboratories, Inc.).

Flow cytometry. Apoptosis was evaluated by flow cytometry using Annexin V-PE and 7-aminoactinomycin D. A Guava Nexin Annexin V Assay kit (Merck KGaA, Darmstadt, Germany) was used according to the manufacturer's protocols. Analysis was performed with the Guava Personal Cell Analysis Flow Cytometer (Guava; EMD Millipore, Billerica, MA, USA) and CytoSoft software (version 2.1.4; Guava; EMD Millipore).

Western blot analysis. Lysates were prepared using radiimmunoprecipitation lysis buffer (Abcam, Cambridge, UK) containing protease inhibitors (Roche Diagnostics, Basel, Switzerland). A biochincnic acid protein assay kit (Thermo Fisher Scientific, Inc.) was used to determine the protein concentration, according to the manufacturer's protocols. Following heating at 97˚C for 5 min, protein samples (50 µg) were subjected to 4-12% SDS-PAGE and transferred to polyvinylidene fluoride membranes at 30 V for 50 min. Membranes were blocked with a matching buffer (20% diluted A, 30% diluted B; WesternBreeze Blocker/Diluent; Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 1 h and incubated with the primary antibodies rabbit anti-HO-1 (dilution, 1:2,000; cat. no., EP1391Y; Abcam) and mouse anti-GAPDH (dilution, 1:1,000; cat. no., AM4300; Ambion; Thermo Fisher Scientific, Inc.) at 4˚C overnight. The following day, the blots were incubated with ready to use secondary antibodies against rabbit (cat. no. WP20007; Invitrogen, Thermo Fisher Scientific, Inc.)
Hyperthermia and cisplatin differentially induce HO-1 mRNA and protein expression in ovarian and gastric cancer cells.

Enhanced expression levels of HO-1 mRNA were only observed in OVCAR-3 cells. The exposure of OVCAR-3 cells to cisplatin resulted in a significant increase of HO-1 mRNA expression. Cisplatin induced a 3.75- and 2.4-fold increase of HO-1 expression in conditions of normothermia (37˚C) and hyperthermia (43˚C), respectively (P<0.05). While hyperthermia at 43˚C boosted HO-1 expression by 1.34-fold, the addition of cisplatin increased the effect on HO-1 expression by 3.22-fold (P<0.05; Fig. 1A). In AGS cells, HO-1 expression was not significantly affected by temperature or cisplatin (Fig. 1B).

Furthermore, cisplatin significantly increased HO-1 protein expression in OVCAR-3 cells (P<0.05). HO-1 expression was increased 9.5-fold, following cisplatin treatment under normothermia. At 43˚C, this effect was slightly higher, with a 9.77-fold increase (P<0.05). The exposure of OVCAR-3 cells to hyperthermia alone had no effect on HO-1 protein expression levels. However, the combination of cisplatin and hyperthermia increased HO-1 protein expression by 11-fold compared with the control (untreated cells in normothermia; P<0.01; Fig. 2A).

In AGS cells, cisplatin had no notable effect on HO-1 expression. At 37˚C, cisplatin increased HO-1 protein expression in AGS cells by 1.2-fold; however, this increase was not statistically significant, whereas the exposure of cells to 43˚C in the absence of cisplatin increased HO-1 protein expression levels by 2.75-fold (P<0.05). HO-1 expression dropped slightly when cisplatin was added at 43˚C. Therefore, concomitant treatment of AGS cells with cisplatin and hyperthermia at 43˚C resulted in a 2.14-fold increase in HO-1 protein compared with the control (Fig. 2B). Furthermore, HO-1 knockdown was assessed by western blotting (Fig. 2C and D).

HO-1-silencing does not influence AGS cell viability. The results of the MTT assay revealed that HO-1-silencing in OVCAR-3 cells does not affect viability in response to cisplatin at 37˚C. The exposure of OVCAR-3 cells to cisplatin and hyperthermia (43˚C) resulted in a 36% drop in cell viability (P<0.05). HO-1-silencing enhanced this effect by an additional 20% (P<0.05; Fig. 3A).

HO-1-silencing in AGS cells enhanced the cisplatin effect and reduced cell viability by 16% at 37˚C (P<0.05).
Hyperthermia potentiated cisplatin cytotoxicity in AGS cells: viability dropped by 24% compared with 37˚C. However, HO-1-silencing had no significant additional effect, whereas viability rates were similar in HO-1-silenced or unsilenced AGS cells following cisplatin treatment at 43˚C (Fig. 3B).

**HO-1-silencing prior to concomitant hyperthermia and cisplatin treatment increases ovarian and gastric cancer cell apoptosis.** The exposure of OVCAR-3 and AGS cells to hyperthermia resulted in a better cell response to cisplatin with respect to apoptosis. Prior HO-1-silencing under normothermia increased cisplatin-induced apoptosis in OVCAR-3 and AGS cells by 2.07- and 2.63-fold, respectively. In addition, silencing of HO-1 under hyperthermia enhanced the apoptosis of OVCAR-3 and AGS cells by 3.09- and 6.84-fold, respectively (P<0.05; Fig. 4).

Hyperthermia enhances the effect of cisplatin on OVCAR-3, but not on AGS cells, following modulation of HO-1 expression. PCR analysis for 48 h following treatment indicated that exposure to cisplatin resulted in a gradual decrease in the cell index of AGS and OVCAR-3 (HO-1-silenced) cells at 37˚C. Hyperthermia at 43˚C boosted this effect by inducing a gradual decrease of the OVCAR-3 (HO-1-silenced) cell index. However, the cell index of AGS (HO-1-silenced) cells following cisplatin treatment at 37 or 43˚C remained similar (Fig. 5).

**Discussion**

In the present study, the HO-1 protein was variably expressed at the basal level and variably induced following exposure to cisplatin and hyperthermia in OVCAR-3 and AGS cells. Cisplatin increased the expression levels of HO-1 in OVCAR-3 cells, while hyperthermia at 43˚C had no effect. In AGS cells, HO-1 expression was slightly increased under hyperthermia, with no significant induction following exposure to cisplatin, indicating that the modulation of HO-1 may serve a role in the response of cancer cells to cisplatin and hyperthermia and affect cancer treatment outcomes.
HIPEC is widely used in clinical settings, and promising results have been reported in the treatment of peritoneal dissemination of gastric and ovarian cancer (29,30). To the best of our knowledge, to date, there has been a lack of evidence regarding the synergy of chemotherapy and hyperthermia. In our previous studies, it was observed that gastrointestinal and ovarian cancer cells responded unpredictably following exposure to cisplatin and hyperthermia (16,31). One of the limits of this response may be the induction of cytoprotective enzymes associated with chemotherapy and/or hyperthermia, in particular HO-1. HO-1 is known to be highly expressed in human gastric and ovarian cancer tissue (32). Anticancer treatment options, including chemotherapy and radiotherapy may increase HO-1 expression (33). HO-1 serves an important role in a number of pathophysiological conditions, including temperature rise and inflammation, and has been reported to be associated with cancer (34,35). HO-1 expression is associated with cancer growth and progression by promoting angiogenesis in the tumor itself and metastases and pro-proliferation in different types of tumors, including renal cell carcinoma, prostate and pancreatic cancer, melanoma, and hepatoma (36-40). Numerous studies have highlighted that cancer cells with high expression levels of HO-1 are less sensitive to cisplatin treatment compared with cancer cells with low HO-1 expression levels (41,42).

The mechanism underlying this cytoprotective effect relies on the ability of HO-1 to catabolize free heme and prevent it from sensitizing cells to undergo programmed cell death (43). HO-1 under normal conditions has various cellular functions, including catalyzing the heme molecule to form bile pigments (44). When stimulus (heat) is present, cellular HO-1 synthesis is enhanced (45). Therefore, the present study suggests that HO-1 is crucially important when dealing with intraperitoneally spread cancer, particularly treating it with HIPEC. Following the administration of heated chemotherapy drugs into the abdominal cavity, tumor cells should start to defend themselves, by activating heat shock proteins, particu-
larly HO-1. The aim of the present study is to achieve better treatment results by downregulating HO-1 expression.

To the best of our knowledge, there are no published data on the efficacy of HIPEC treatment while modulating the expression of HO-1. The results of our study demonstrate the impact of HO-1 expression modulation in the combination treatment of hyperthermia at 43°C and cisplatin in OVCAR-3 cells.

Zhao et al (46) reported that the basal level of HO-1 expression is higher in ovarian cancer cells compared with normal ovarian tissues. A high level of HO-1 expression has also been associated with aggressive tumors and poor clinical outcomes (46). The ability of cisplatin to increase the expression of HO-1 was also observed in different cancer types, including pancreatic and hepatic cancer (43,47,48). Was et al (37) reported the different abilities of tumor tissues to produce heat shock proteins. Nonetheless, a high level of HO-1 is known to be associated with the reduced tumor growth observed in some types of cancers, including breast and prostate cancer and non-small-cell lung carcinoma (37).

The results of the present study indicate that the viability of HO-1-silenced OVCAR-3 cells decreased significantly following cisplatin treatment at 43°C. However, in AGS cells, the inhibition of HO-1 did not improve the response to cisplatin treatment. These results are associated with the expression of HO-1. It is possible that the inhibition of HO-1 only increases the effect of cisplatin in cancer cells, where HO-1 is highly expressed. This is in accordance with the data reported by Lx et al (41), where cisplatin significantly induced the expression of HO-1. The study modulated HO-1 expression using hemin (an inducer of HO-1) and ZnPPIX (an inhibitor of HO-1), and reported that hemin strongly inhibited cisplatin-induced cell death, while ZnPPIX significantly increased cell death (41,49). These effects following HO-1 modulation can be explained by the cytoprotective ability of this protein. HO-1 activates a cellular defense mechanism against oxidative stress through its catalytic products, including ferrous iron, carbon monoxide, and biliverdin (37). In addition, growing evidence has suggested that HO-1 protects cells from chemotherapeutic agent-induced apoptosis, and the targeted knockdown of HO-1 gene expression or suppression of HO-1 activity in vitro significantly enhances the chemosensitivity of cancer cells (50). Furthermore, it has been reported that the inhibition of HO-1 can increase cellular response to anticancer treatment (26).

Cisplatin can effectively induce and promote apoptosis in a wide range of solid tumors, including head and neck cancer, esophageal carcinoma, non-small cell lung carcinoma, and testicular, cervical, and ovarian cancer (51). Inhibition of HO-1 may strengthen the pro-apoptotic effects of cisplatin (41). In the present study, the inhibition of HO-1 increased the number of apoptotic cells in OVCAR-3 and AGS cell lines, however these results did not indicate any significant differences associated with HO-1 expression and cell viability. This could be explained by the fact that the present study measured the number of cells in both early and late apoptosis, and early apoptosis can be reversible (52). Geske et al (52) reported that the early stages of apoptosis are reversible if the apoptotic stimulus is removed, which is the reason that PCR analysis was performed in the present study.

In this experimental model, hyperthermia alone did not induce the upregulation of HO-1 expression in the tested cancer cell lines. Nevertheless, HO-1-silencing resulted in the optimal response to cisplatin treatment in terms of cell viability in OVCAR-3 cells, and apoptosis in both OVCAR-3 and AGS cells, under conditions of hyperthermia. Therefore, a novel finding regarding the role of HO-1 in HIPEC is presented in this study. In conclusion, the cytoprotective protein HO-1 is induced in cancer cells by different stressors in a variable manner. In tumors with highly inducible HO-1, the present study indicated that prior silencing of this gene may significantly improve the cellular response to hyperthermia and cisplatin.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

VC wrote the manuscript and analyzed the data. AS performed the western blot analysis and analyzed the data. GS and AJ performed semi-quantitative PCR and siRNA transfection. SP performed the PCR cell analysis and analyzed the data. ZD and AG revised the data and manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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