Dihydroartemisinin promotes CHAC1 transcription to induce ferroptosis in primary liver cancer cells: activation of unfolded protein responses

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Abstract

Background: This study aimed to explore whether dihydroartemisinin (DHA), an artemisinin derivate drug, eliminates primary liver cancer (PLC) cells by inducing ferroptosis.

Methods: Four PLC cell lines were treated with varied concentrations of DHA. RNA interference was performed to knock down the expression of unfolded protein response (UPR) sensors *in vitro*.

Results: DHA-caused PLC cell death was irrelevant to p53 status. PLC cells exposed to DHA displayed classic ferroptosis features – increased lipid ROS, MDA and iron ions, and decreased activity or expression of GSH, GPX4, SLC7A11 and SLC3A2. The anti-tumor effects of DHA were significantly weakened by ferrostatin-1 and deferoxamine mesylate salt, but augmented by iron overload. DHA activated all three UPR branches, including PERK/eIF2/ATF4, IRE1 α / XBP1 , and ATF6, *in vitro* . Further, to deactivate UPRs, exclusive siRNA was used to silence the expression of ATF4, XBP1 or ATF6 in PLC cells. Unexpectedly, ferroptosis induced by DHA was significantly attenuated when ATF4, XBP1 or ATF6 was knocked down. The transcription of CHAC1, a molecule that is capable of degrading GSH, was enhanced by DHA, but weakened when the above three UPR transcription factors were silenced.

Conclusion: DHA effectively induces ferroptosis in PLC cells, which involves the activation of anti-survival UPRs.

Background

Primary liver cancers (PLCs), consist predominantly of hepatocellular carcinoma (HCC), is after lung cancer the second leading cause of cancer-related death worldwide [1, 2]. The morbidity of PLCs markedly varies between geographic regions around the world, and is highest in sub-Saharan Africa and Eastern Asia [3]. The major risk factors of PLCs include chronic infection of hepatitis B/C viruses, excess alcohol consumption, non-alcoholic fatty liver disease, and aflatoxin exposure [3, 4]. Although current treatments, including percutaneous local ablation, surgical resection, and liver transplantation, improve the survival of patients with PLCs, PLC-correlated mortality still increases owing to population growth and high recurrence [1, 5, 6]. Therefore, to develop novel therapies for PLCs based on molecular studies is urgently needed.

Ferroptosis is an iron-dependent form of non-apoptotic cell death first reported by Dr. Stockwell's group in 2012 [7]. This type of cell death is caused by lethal lipid peroxidation, and has typical features distinct from other types of regulated cell death, such as overproduction of lipid reactive oxygen species (ROS), impairment of crista and outer mitochondrial membrane [8]. One of the major reasons for therapy resistance in cancer is intrinsic or acquired resistance of cancer cells to executioner-mediated apoptosis [9]. By comparing to non-cancer cells, cancer cells often demand increased iron to enable their growth. Interestingly, this iron dependency also makes cancer cells more sensitive to ferroptosis [10]. Therefore, small molecules capable of inducing iron-dependent accumulation of lipid ROS, such as erastin, sorafenib and Ras-selective lethal small molecule (RSL)-3/-5 are being evaluated for their potential in

cancer therapy [11, 12]. To identify drugs capable of inducing ferroptosis will provide insights for the potential of ferroptosis as a new promising target for PLC treatment.

Artemisinin is a sesquiterpene trioxane lactone originally extracted from *Artemisia annua* L, and its derivatives are effective anti-malarial agents [13]. Currently, besides the well-known application in anti-malaria field, artemisinins are being evaluated for treating multiple cancers due to their established safety recorded in thousands of malarial patients [13]. Ooko and colleagues treated 60 cancer cell lines with 11 artemisinin derivatives, and analyzed the expression profiles of 30 iron-related genes in these cell lines via microarray hybridization [14]. They found that the $\log_{10}$ (50% inhibition concentration, IC_{50}) values of analyzed artemisinins significantly correlated to the expression of at least 20 iron-related genes [14]. Their findings imply the involvement of artemisinins in ferroptosis of cancer cells. The most frequently reported derivative of artemisinin that triggers ferroptosis in malignant cells is artesunate [15, 16]. Recently, dihydroartemisinin (DHA), a semisynthetic derivative of artemisinin, has been demonstrated to exhibit anti-tumor activity in PLCs *in vitro* and *in vivo* [17, 18]. The role of DHA in inducing ferroptosis in cancer cells was first reported in head and neck carcinoma cells by Lin *et al.* [19], and later in leukemia cells by Du *et al.* [20] and in glioma cells by Chen *et al.* [21]. Up to date, no direct evidence has proved DHA's role in inducing ferroptosis in PLCs. Nonetheless, an earlier study from Wang and co-workers has shown that DHA is able to increase intracellular ROS in LM3 HCC cells [17], suggesting that DHA may trigger ferroptosis in PLC cells.

Cancer cells can thrive under hostile microenvironmental conditions. Within tumor masses, endoplasmic reticulum (ER) stress is provoked by nutrient deprivation, oxygen limitation and high metabolic demand [22]. Malignant cells initiate unfolded protein response (UPR) to cope with ER stress [23]. Three ER-resident sensors, protein kinase R-like ER kinase (PERK), inositol-requiring transmembrane kinase/endonuclease 1 α (IRE1 α) and activating transcription factor (ATF) 6, coordinate UPR if misfolded proteins accumulate and aggregate beyond a tolerable threshold [22, 23]. ATF4 upregulation induced by ferroptosis inducers, such as Erastin and Artesunate, is considered as a compensatory effect, and its knockdown is demonstrated to further augment ferroptosis in cancer cells [15, 24]. In glioma cells, PERK/ATF4 signaling pathway was activated by DHA and negatively regulated DHA-induced ferroptosis [21]. Studies on revealing the roles of ATF6- and IRE1 α -mediated UPR branches in ferroptosis are scarce, and how DHA affects these two UPR mediators remains largely unknown.

In this study, four PLC cell lines, Hep3B, Huh7, PLC/PRF/5 and HepG2, were treated with different concentrations of DHA in presence or absence of ferroptosis inhibitors or iron ions. Our results demonstrated that DHA was capable of inducing ferroptosis and activating all the three UPR branches in PLC cells. Interestingly, our data for the first time demonstrated that DHA-induced ferroptosis was weakened if these UPR signaling pathways were suppressed.

Materials And Methods

Chemicals

Ferrostatin-1 and DHA both from Aladdin (Shanghai, China) were dissolved in dimethyl sulfoxide (DMSO) into a stock concentration of 5 mM and 50 mM, respectively. Deferoxamine mesylate salt (DFOM; MedChemExpress, Monmouth Junction, NJ, USA) and iron chloride hexahydrate (Aladdin) were dissolved in distilled H₂O into stock concentrations of 50 mM and 2 mg/L, respectively.

Cell culture and transfection

PLC cell lines, including Hep3B (p53 null), Huh7 (659 A>G *TP53* mutant), PLC/PRF/5 (*TP53* 747 G>T mutant) and HepG2 (p53 wild-type), were provided by Cell Bank of Chinese Academy of Sciences (CBCAS, Shanghai, China). The short tandem repeat (STR) of each cell line was confirmed correct. No mycoplasma contamination was detected. Hep3B, PLC/PRF/5 and HepG2 cells were maintained in MEM (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), while Huh7 cells were cultured in DMEM supplemented with 10% FBS. Cells were maintained in an HF-90 cell incubator (Lishen, Shanghai, China) in an atmosphere of 5% CO₂ at 37 °C. PLC cells were transfected with siATF4 (Forwards 5'-3', guccuccacuccagaucautt; Reversed 5'-3', augaucuggaguggaggactt), siXBP1 (Forwards 5'-3', gcaagugguagauuuagaatt; Reversed 5'-3', uucuaaaucuaccacuugctt), siATF6 (Forwards 5'-3', gaaaugucgguucagauaatt; Reversed 5'-3', auaucugaaccgacauuuctt) or siCHAC1 (siRNA-1 Forwards 5'-3', gacgcuccuugaagaucatt, Reversed 5'-3', augaucuugaaggagcguctt; siRNA-1 Forwards 5'-3', gccacaaccuugaauacuutt, Reversed 5'-3', aaguaaucaagguuguggctt) for 24 hrs, and then treated with DHA for additional 24 hrs.

Cell viability assay

PLC cells were treated with different concentrations of DHA, and their viabilities were determined with CCK-8 (Sigma-Aldrich, St. Louis, MO, USA). In short, PLC cells were placed into 96-well plates at a density of 3×10^3 /well, and 24 hrs later, they were treated with 2, 5, 7.5, 10, 20, 30, 40 or 50 μ M DHA for 24 hrs. Then, cells were incubated with 10 μ L CCK-8 for 1 hr, and the absorbances at 450 nm were determined with a microplate reader (BioTek, Winooski, VT, USA).

PLC cell xenografted tumor mouse models

Male nude mice (BALB/c) of 6-8 weeks old (18-20g) were obtained from HFK Bioscience (Beijing, China), and housed in specific pathogen free facility. A total of 32 mice were subjected into the xenograft mouse model experiment, and randomly divided into four groups (n = 8/group). PLC cells were subcutaneously injected into the nude mice. The tumor volumes were determined by using the following formula: $0.5 \times \text{tumor length} \times (\text{tumor width})^2$. When the xenografted tumor grew into a size of approximately 80-100 mm³, half mice in each group were given 100 mg/kg DHA for 5 d per week by gavage. Twenty-one days later, all mice were sacrificed by isoflurane anaesthesia and cervical dislocation before collecting the tumor tissues.

Hematoxylin and eosin (H&E) staining

Tumor tissues were collected, fixed in 4% paraformaldehyde, and embedded into paraffin. The tissue blocks were sliced into 5- μ m sections. Following the deparaffinating and rehydration, the samples were incubated with H&E staining agents according to the manufacturer's protocols.

ROS measurements

Contents of total ROS and lipid ROS were determined with an ROS assay kit (NJJC Bio, Nanjing, China; Invitrogen, Carlsbad, CA, USA) based on DCFH-DA and BODIPY 581/591 C11 fluorescence as per the supplier's protocols. The DCF and C11-BODIPY fluorescence intensities were analyzed with the Tecan M200 PRO automatic microplate reader or a flow cytometer.

Iron concentration

Intracellular ferrous iron levels were analyzed by using an iron assay kit obtained from Leagene Biotech. (Beijing, China) according to the manufacturer's instructions. The output was measured on the Tecan M200 PRO reader at optical density (OD) of 562 nm.

Malondialdehyde (MDA), GSH levels, and GSH-PX activity

According to the manufacturer's instructions, A003-1, A061-1 and A005 kits (all from NJJC Bio) were used to determine MDA levels, GSH levels and GSH-PX activity, respectively.

Reverse transcriptional PCR (RT-PCR)

Total RNAs were isolated from PLC cells treated with DHA for 1, 6, 12, or 24 hrs. The splicing of XBP1 mRNA (NM_005080 and NM_001079539) was assessed with RT-PCR. One pair of primers (forward, 5'-3' aaacttttgctagaaaatcagc; reverse, 5'-3' caataccgccagaatcca) that spanned the spliced site was used for RT-PCR. Two fragments (252 bp and 226 bp) can be detected. PCR products were analyzed with agarose gel.

Western blotting analysis

Total proteins from the whole cell lysates were isolated by using RIPA lysis buffer containing 1% PMSF (SolarBio, Beijing, China). Nuclear proteins were isolated with a Nuclear and Cytoplasmic Protein Extraction kit (Beyotime, Shanghai, China). Protein concentrations were analyzed with a BCA kit (SolarBio). After separating via SDS-PAGE, proteins were transferred to PVDF membranes (Millipore, Billerica, MA, USA), and blocked with 5% non-fat milk for 1 hr. The membranes were treated with primary

antibodies at 4°C overnight, and then with secondary antibodies at 37°C for 1 hr. The information of primary antibodies were shown in Table 1. Colors of protein blots were developed by incubating the membranes with ECL agent (SolarBio).

pGL3 luciferase reporter assay

The promoter (-2000bp to +30 bp) of *CHAC1* gene was inserted into pGL3 luciferase reporter, and the promoter activity was determined by analyzing Firefly/Renilla luciferase ratio according to the manufactory's instructions (Promega, Madison, WI, USA)

Statistical analysis

Data were expressed by mean values $\pm$ standard deviation. GraphPad Prism version 8.0 software was utilized to compare the data. One-way analysis of variance (ANOVA) and two-way ANOVA followed by Bonferroni's multiple comparison test were used to compare data from multiple groups. A p-value < 0.05 was considered significant.

Results

DHA induced-ferroptosis in PLC cells is irrelevant to p53 status

Considering the involvement of p53 in ferroptosis [25], four PLC cell lines with discrepant p53 statuses, Hep3B (p53 null), Huh7 and PLC/PRF/5 (both p53 mutant) and HepG2 (p53 wild-type), were treated with DHA of increased concentrations for 24 hrs. Our data showed that DHA was able to inhibit the survival of all analyzed PLC cell lines, irrelevant to their p53 statuses (Fig. 1). Two different ferroptosis inhibitors, DFOM (an iron chelator; 10 μ M for Huh7 cells and 50 μ M for the others) and ferrostatin-1 (a lipid peroxidation inhibitor; 5 μ M for HepG2 and 1 μ M for the others) were utilized to treat PLC cells in presence of DHA. The concentrations of DFOM and ferrostatin-1 used here were determined by the preliminary experiments. We found that the cytotoxic effects of DHA on PLC cells were attenuated by both ferroptosis inhibitors (Fig. 1). In contrast, addition of exogenous iron ions (10 μ g/mL iron chloride hexahydrate) further augmented DHA's effects (Fig. 1).

The IC_{50} values of DHA were 29.4 ± 1.7 μ M for Hep3B, 32.1 ± 4.5 μ M for Huh7, 22.4 ± 3.2 μ M for PLC/PRF/5, and 40.2 ± 2.1 μ M for HepG2. These findings suggested that the resistance of these cell lines to DHA was HepG2 > Huh7 > Hep3B > PLC/PRF/5. On the basis of the IC_{50} values, HepG2, Huh7, Hep3B and PLC/PRF/5 were further treated with 40 μ M, 35 μ M, 30 μ M and 25 μ M DHA, respectively.

DHA induces ferroptotic GSH synthesis in PLC cells

After incubation with DHA for 1, 6, 12 or 24 hrs, PLC cells were harvested to analyze total and lipid ROS content (Fig. 2a-b), MDA levels (Fig. 2c), and iron concentrations (Fig. 2d). The results showed increased ROS, MDA and iron levels in PLC cells exposed to DHA. These above data collectively depicted DHA as ferroptosis inducer in PLC cells *in vitro*.

Blocking GSH synthesis is known to facilitate toxic ROS accumulation [7]. Therefore, we analyzed GSH contents with a commercial available kit in DHA-treated PLC cells. As compared to the blank cells, GSH/GSSG (oxidized form of GSH) ratios markedly decreased in cells exposed to DHA (Fig. 2e). The expression of GPX4, SLC7A11 and SLC3A2, and the activity of GSH-PX were inhibited by DHA as well (Fig. 2e-f). In contrast, CHAC1 expression increased in response to DHA treatment (Fig. 2f). These data demonstrate DHA as a negative regulator for GSH synthesis in PLC cells.

DHA limits xenografted PLC tumor growth *in vivo*

We next investigated the effects of DHA on PLC growth *in vivo*. No weight loss or health problem was observed in mice. PLC cells were subcutaneously injected into immune-deficient mice. DHA was given to these mice when the tumor volumes reached 80–100 mm³. The tumor volumes were recorded every three days, and three weeks later, the xenografted tumors were collected. As shown in Fig. 3a-c, DHA limited, but did not terminate, the formation of xenografted PLCs. Further, DHA augmented ROS accumulation, and downregulated GSH-PX activity in the tumor masses. These data together with earlier results from cell experiment confirmed that DHA could induce ferroptosis both *in vitro* and *in vivo*.

DHA activates three UPR branches in PLC cells

As DHA induced ferroptosis in all analyzed PLC cells *in vivo* and *in vitro*, we determined the alterations in UPR-associated molecules only in PLC/PRF/5 and HepG2 cells. Our data indicated that all the three UPR signaling pathways were activated by DHA—the expression of phosphorylated PERK, eIF2 α , and IRE1 α increased, and the levels of ATF4, nuclear ATF6 and spliced XBP1 upregulated (Fig. 5). DHA activated PERK/eIF2 α /ATF4 signaling earlier in PLC/PRF/5 cells than in HepG2 cells (Fig. 4). To determine the role of activated UPR in DHA-mediated ferroptosis, ATF4, XBP1 and ATF6 were respectively silenced with their exclusive siRNAs. PLC cell vitalities increased (Fig. 5a & d), total ROS (Fig. 5b & e) and lipid ROS (Fig. 5 c & d) decreased and when the three UPR sensors were knocked down. The expression levels of GPX4, SLC7A11, SCL3A2 and CHAC1 were restored to different degrees in cells when UPR transcription factors were suppressed (Fig. 6a-f). These data for the first time demonstrated DHA-induced UPR activation as an anti-survival mechanism in PLC cells.

DHA promotes the transcription of CHAC1 by activating UPRs in PLC cells

The potential binding sites for ATF4, XBP1 and ATF6 on *CHAC1* gene promoter were predicted with JASPER (<http://jaspar.genereg.net/>) and PROMO (http://algggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3). Multiple sites on *CHAC1* promoter could be binding by these transcription factors (Fig. 7a). Since DHA could effectively increase the expression of ATF4, XBP1 and ATF6, it is possible that this agent can promote *CHAC1* transcription. Results from pGL3 luciferase reporter assay confirmed that DHA enhanced the promoter activity of *CHAC1* gene (Fig. 7b). In addition, to further validate the role of CHAC1 in DHA-induced ferroptosis, siRNA was used to knock CHAC1 expression down (Fig. 7 d & e). We found that ferroptosis induced by DHA was suppressed by CHAC1 siRNAs (Fig. 7 f-i).

Discussion

Inducing ferroptosis is being explored as an alternative approach to eradicate cancer cells resistant to apoptosis [10]. Several artemisinin derivatives have been demonstrated to kill cancer cells by inducing ferroptosis [14]. A few recent studies revealed DHA as ferroptosis inducer in cancer cells [19-21]. In consistent with these previous studies, our work demonstrated that DHA killed PLC cells by inducing ferroptosis (sFig. 8).

The classic way to determine whether a drug can induce ferroptosis is to co-treat cancer cells with ferroptosis blockers or iron ions [11, 12]. The anti-tumor effects of ferroptosis inducers, such as erastin and sorafenib, can be attenuated by iron chelators (such as DFOM) and inhibitors of lipid peroxidation inhibitor (such as ferrostatin-1), but augmented by adding exogenous iron ions [7, 26]. Based on the IC₅₀ results, we concluded that the PLC cells were more resistance to DHA-induced cytotoxicity when ferroptosis was suppressed by DFOM or ferrostatin-1. In contrast, addition of iron chloride hexahydrate sensitized PLC cells to DHA. Of note, treatment of DFOM or ferrostatin-1 does not completely abolish the anti-tumor effects of DHA, suggesting that triggering ferroptosis is not the only way for DHA to induce cytotoxicity in PLC cells. Although DHA has been shown to induce ferroptosis in other types of cancer cells [19-21], our work demonstrates such effects in PLC cells for the first time.

p53 is a key tumor suppressor, whose mutation or loss directly contributes to tumorigenesis [27]. SLC7A11 (also known as xCT) and SLC3A2 controls the import of extracellular cysteine and cystine, and regulates GSH synthesis [28, 29]. Interestingly, p53 of wild-type and acetylation-defective mutant can suppress SLC7A11 expression, thereby inhibiting GSH synthesis and sensitizing tumor cells to ferroptosis [25]. In light of the key role of p53 in SLC7A11-associated ferroptosis, we determined the cytotoxicity of DHA in four PLC cell lines with different p53 statuses. Our data demonstrated that DHA could effectively induce ferroptosis in the analyzed PLC cells, even in the p53 null cells (Hep 3B cells). It is worth noting that DHA also exhibited anti-tumor activity by activating Caspase-3, a key apoptosis regulator, against PLCs, regardless of p53 status [30]. This study together with our work suggests that DHA exerts its anti-tumor effects in an p53 independent manner. Thus, DHA is an attractive drug for treating p53 mutation- or deficiency-associated cancer.

GPX4 was first identified by Urisini et al. in 1982 [31], and is now recognized to possess a unique capability of reducing reactive phosphatidylcholine hydroperoxides and suppressing lipid peroxidation [8]. Direct or indirect targeting mechanism such as GSH depletion induces GPX4 repression [32]. In cancer cells, ferroptosis can be provoked by GPX4 inactivation [10]. To further explore how DHA induces ferroptosis in PLC cells, the contents of GSH and the expression and activity of GPX4 were analyzed. Our data showed that DHA reduced GSH synthesis and inhibited GPX4 expression in PLC cells. The decreased activity of GPX4 observed in PLC cells exposed to DHA may result from the reduced GPX4 expression. While a study from Lin et al [19]. supported our findings, a recent work from Chen and co-workers did not [21]. The latter study demonstrated that DHA induced ferroptosis in glioma cells and it also induced a compensatory upregulation of GPX4 in U251 and U373 cancer cells [21]. At present, we cannot find a reasonable explanation for such paradoxical phenomenon. Nonetheless, these findings, at least, suggest that DHA's effects on regulating GPX4 expression in different solid tumors are inconsistent. The mechanisms underlying such interesting findings require to be further investigated.

Next, we explored whether UPR-activated by DHA played a role in ferroptosis. UPR is coordinated by PERK/eIF2 α /ATF4, IRE1 α /XBP1 and ATF6 branches [22, 23]. In response to ER stress, PERK is self-phosphorylated to induce the phosphorylation of eIF2 α , thereby promoting ATF4 translation [33]. Once phosphorylated, IRE1 α splices XBP1 into a short form [33]. ATF6 translocates into cell nucleus to function as a transcription factor after cleaving from ER [34]. ATF4 is suggested a protective molecule against ferroptosis because of its ability to activate the transcription of SLC7A11 [24]. Our prior work also demonstrated ATF4 as a pro-survival factor in erastin-induced ferroptosis in PLC cells (accepted). Therefore, we assumed that blocking PERK/eIF2 α /ATF4 signaling pathway would further augment the anti-tumor effects of DHA in PLC cells. However, by determining cell vitality and ROS contents, we unexpectedly found that knockdown of ATF4 attenuated DHA's cytotoxic effects. GPX4 expression was upregulated in response to ATF4 silencing. Interestingly, we also found that DHA-triggered ferroptosis was attenuated when XBP1 or ATF6 was silenced. Unlike most previous studies revealing anti-ferroptosis roles of these UPR proteins [21, 24], our work demonstrates them as pro-ferroptosis molecules in DHA-treated PLC cells (sFig. 8).

Due to the significant role of ATF4 in inducing SLC7A11 expression [24], it is hard to find previous evidence to support the pro-ferroptosis role of ATF4 observed in our study. Nonetheless, an earlier study evaluating the effects of artesunate, another classic ferroptosis inducer, in Burkitt's lymphoma cells [15] indirectly supported our findings. This study showed that ATF4 activated the transcription of CHAC1 to augment ferroptosis induced by artesunate in DAUDI and CA-46 cells [15]. CHAC1 function as γ -glutamyl cyclotransferase to induce GSH degradation [35]. Such findings imply that ATF4 upregulation also leads to GSH degradation. It is possible that DHA, like artesunate, activates mechanisms important for GSH degradation mediated by UPR proteins, rather than GSH synthesis. By performing dual luciferase reporter assay, we demonstrated that DHA enhanced the promoter activity of *CHAC1* gene. Unlike ATF4, to our knowledge, XBP1 and ATF6 were demonstrated to promote CHAC1 expression for the first time.

The first limitation of our study was that we only knocking down the expression of key UPR regulators, ATF4, XBP1 and ATF6, in PLC cells. Since the endogenous expression levels of them are already abundant in PLC cells stimulated with DHA, our group believe that knocking them down is more appropriate. Nonetheless, to demonstrate the roles of activated UPR in DHA-induced ferroptosis, overexpressing them in PLC cells is still needed. There are several binding sites predicted for XBP1 and ATF6 on CHAC1 promoter. Although the emphasis of this study is to demonstrate how DHA affects the promoter activity of CHAC1, there is still a need to identify the specific locations that these UPR sensors bind to. This is the second limitation.

Conclusion

Our study demonstrates that DHA effectively induces ferroptosis and activates UPRs in PLC cells. Inhibition of UPRs suppresses the cytotoxic effects of DHA on PLC cells, which involved a reduction in CHAC1, a key gene responsible for GSH degradation. DHA promotes the transcription of CHAC1. These findings provide novel insights into the anti-tumor effects of DHA, suggesting UPR triggered by DHA is pro-death in PLC cells.

Abbreviations

DHA, dihydroartemisinin

PLC, primary liver cancer

ROS, reactive oxygen species,

MDA, malondialdehyde

GSH, glutathione

GPX4, glutathione peroxidase 4

PERK, protein kinase R-like ER kinase

eIF2, eukaryotic initiation factor 2

ATF4, activating transcription factor 4

ATF6, activating transcription factor 6

IRE1 α , inositol-requiring transmembrane kinase/endoribonuclease 1 α

XBP1, X box-binding protein 1

CHAC1, ChaC Glutathione Specific Gamma-Glutamylcyclotransferase 1

SLC7A11, Solute Carrier (SLC) Family 7 Member 11

SLC3A2, Solute Carrier (SLC) Family 3 Member 2

Declarations

Ethics approval

The present work was approved by the First Affiliated Hospital of Zhengzhou University, and the animal procedures conformed to *the Guideline for the Care and Use of Laboratory Animals*.

Consent to participate

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

ZW and TB designed the study, performed the experiments and analyzed the data. ZW drafted the manuscript. ML, YL and ZQ, performed the experiments and helped with data analysis. LY and BL performed the experiments and revised the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1 Antibodies used in western blotting analysis.

Antibodies	Suppliers	Catalog number	Dilution
ATF4	CST, Danvers, MA, USA	11815	1:1000
SLC7A11		12691	1:1000
ATF6	ABclonal, Wuhan, China	A0202	1:1000
p-eIF2α		AP0692	1:100
eIF2α		A0764	1:500
GPX4	Abcam, Cambridge, MA, USA	AB125066	1:3000
SLC3A2		AB108300	1:5000
CHAC1		AB76386	1:1000
p-IRE1α	Affinity Biosciences, Cincinnati, OH, USA	DF8322	1:1000
IRE1α		DF7709	1:1000
PERK		AF5304	1:1000
p-PERK	ThermoFisher Scientific, Pittsburgh, PA, USA	PA5-40294	1:500

ATF4, activating transcription factor 6

PERK, protein kinase R-like ER kinase

p-PERK, phosphorylated PERK

eIF2 α , eukaryotic initiation factor 2 α

p-eIF2 α , phosphorylated eIF2 α

IRE1 α , inositol-requiring transmembrane kinase/endoribonuclease 1 α

p-IRE1 α , phosphorylated IRE1 α

GPX4, glutathione Peroxidase 4

CHAC1, ChaC Glutathione Specific Gamma-Glutamylcyclotransferase 1

SLC7A11, Solute Carrier (SLC) Family 7 Member 11

SLC3A2, Solute Carrier (SLC) Family 3 Member 2

Figures

concentration. The vitality of PLC cells were determined with CCK-8 assay (A, n = 3). IC50 values were calculated (B, n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 vs DHA. Values were presented as mean values ± standard deviation. DHA, dihydroartemisinin; PLC, primary liver cancer

Figure 2

DHA augments ROS generation, and upregulates MDA and iron levels in PLC cells Based on IC50 values, HepG2, Huh7, Hep3B and PLC/PRF/5 were treated with 40 μM, 35 μM, 30 μM and 25 μM DHA,

respectively. After incubation with DHA for 1, 6, 12 or 24 hrs, total ROS (a) and lipid ROS generation (b), MDA levels (c), iron concentrations (d), GSH/GSSG ratios (e), and GSH-PX activity (f), and the protein expression of GPX4, SLC7A11, SLC3A2 (g) of PLC cells were determined (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 vs control (0 h). Values were presented as mean values $\pm$ standard deviation. DHA, dihydroartemisinin; ROS, reactive oxygen species; MDA, malondialdehyde; PLC, primary liver cancer; GPX4, glutathione peroxidase 4; CHAC1, ChaC Glutathione Specific Gamma-Glutamylcyclotransferase 1; SLC7A11, Solute Carrier (SLC) Family 7 Member 11; SLC3A2, Solute Carrier (SLC) Family 3 Member 2

Figure 3

Administration of DHA limits PLC tumor growth in nude mice PLC cells were subcutaneously injected into immune deficient mice. DHA (100 mg/kg/d, 5 d/wk) was given to these mice when the tumor volumes reached 80–100 mm³. The tumor volumes were recorded every three days (a-b), and three weeks later, the xenografted tumors were collected for H&E staining (c; bar, 100 μ m). The ROS contents (d) and GSH-PX activities (e) in tumor tissues were determined (n = 4). *p < 0.05, **p < 0.01, ***p < 0.001 vs control. Values were presented as mean values $\pm$ standard deviation. DHA, dihydroartemisinin; PLC, primary liver cancer; ROS, reactive oxygen species; GSH-PX, glutathione peroxidase

Figure 4

DHA activates UPRs in PLC cells in vitro. HepG2 and PLC/PRF/5 were treated with 40 μ M and 25 μ M DHA for 1, 6, 12 or 24 hrs. The cellular proteins were extracted to analyze the protein levels of UPR-associated molecules via western blot analysis (a & c). Total RNAs were isolated to analyze the formation of sXBP1 in PLC cells via RT-PCR (b & d). DHA, dihydroartemisinin; PLC, primary liver cancer; UPR, unfolded protein response; PERK, protein kinase R-like ER kinase, eIF2, eukaryotic initiation factor 2; ATF 4, activating

transcription factor 4; ATF 6, activating transcription factor 6; IRE1α, inositol-requiring transmembrane kinase/endoribonuclease 1α; unXBP1, unsliced X box-binding protein 1; sXBP1, sliced X box-binding protein 1

Figure 5

The cytotoxic effects of DHA are attenuated when the UPRs are blocked Three specific siRNAs were synthesized to target ATF4, XBP1 and ATF6 in PLC cells. Twenty-four post the siRNA transfection, HepG2

and PLC/PRF/5 were treated with 40 μ M and 25 μ M DHA for 24 hrs. Cell vitalities were determined with CCK8 assay (a & d), and intracellular total ROS (b & e) and lipid ROS (c & f) levels were determined with flow cytometry (b-c, e-f) (n = 3). ***p < 0.001 vs DHA + siNC. Values were presented as mean values $\pm$ standard deviation. DHA, dihydroartemisinin; PLC, primary liver cancer; UPR, unfolded protein response; PERK, protein kinase R-like ER kinase, eIF2, eukaryotic initiation factor 2; ATF4, activating transcription factor 4; ATF6, activating transcription factor 6; IRE1 α , inositol-requiring transmembrane kinase/endoribonuclease 1 α ; unXBP1, unsliced X box-binding protein 1; sXBP1, sliced X box-binding protein 1; siNC, negative control siRNA

Figure 6

Knockdown of UPRs partly alters the expression of molecules associated with ferroptosis in PLC cells in presence of DHA. Three specific siRNAs were synthesized to targeting ATF4, XBP1 and ATF6 in PLC cells. Twenty-four post the siRNA transfection, HepG2 and PLC/PRF/5 were treated with 40 μ M and 25 μ M DHA for 24 hrs. The cellular proteins were extracted to analyze the protein levels of UPR-associated molecules via western blot analysis (a-f). Total RNAs were isolated to analyze the formation of sXBP1 in PLC cells

via RT-PCR (b & e). DHA, dihydroartemisinin; PLC, primary liver cancer; UPR, unfolded protein response; PERK, protein kinase R-like ER kinase, eIF2, eukaryotic initiation factor 2; ATF4, activating transcription factor 4; ATF6, activating transcription factor 6; IRE1 α , inositol-requiring transmembrane kinase/endoribonuclease 1 α ; unXBP1, unsliced X box-binding protein 1; sXBP1, sliced X box-binding protein 1; CHAC1, ChaC Glutathione Specific Gamma-Glutamylcyclotransferase 1; SLC7A11, Solute Carrier (SLC) Family 7 Member 11; SLC3A2, Solute Carrier (SLC) Family 3 Member 2

Figure 7

DHA enhances the promoter activity of CHAC1 gene (a) Potential binding sites for ATF4, XBP1 and ATF6 on CHAC1 gene promoter. (b) pGL3 luciferase reporter assay was carried out to determine the promoter activity of CHAC1 gene. (c) Two siRNAs were used to knock CHAC1 expression down in PLC cells. The protein levels of GPX4 and CHAC1 were determined with Western blot. Cell vitalities were determined with CCK8 assay (f & g), and intracellular lipid ROS (h & i) levels were determined with flow cytometry (n = 3). Values were presented as mean values $\pm$ standard deviation. ATF4, activating transcription factor 4; ATF6, activating transcription factor 6; XBP1, X box-binding protein 1; CHAC1, ChaC Glutathione Specific Gamma-Glutamylcyclotransferase 1

Figure 8

DHA activates ferroptosis by promoting UPR-mediated CHAC1 expression in PLC cells. DHA exerted iron-independent cytotoxic effects in PLC cells, and provoked the activation of all three UPRs, including PERK/eIF2/ATF4, IRE1α/XBP1 and ATF6. RNAi was performed to knock ATF4, XBP1 and ATF6 down. Additional data revealed that inhibition of UPRs attenuated ferroptosis induced by DHA. DHA activated ferroptosis by promoting UPR-mediated CHAC1 expression in PLC cells. DHA, dihydroartemisinin; PLC, primary liver cancer; PERK, protein kinase R-like ER kinase; eIF2, eukaryotic initiation factor 2; ATF4,

activating transcription factor 4; ATF6, activating transcription factor 6; IRE1 α , inositol-requiring transmembrane kinase/endoribonuclease 1 α ; XBP1, X box-binding protein 1; CHAC1, ChaC Glutathione Specific Gamma-Glutamylcyclotransferase 1