Combination of Palmitic Acid and Methylseleninic Acid Induces Mitochondria-Dependent Apoptosis via Attenuation of the IRE1α Arm and Enhancement of CHOP in Hepatoma

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ABSTRACT: The most common saturated fatty acid in the human diet is palmitic acid (PA), and emerging evidence suggests that it may have anticancer activity. Methylseleninic acid (MSeA), the most commonly used selenium derivative in humans, has specific cytotoxic effects on several cancer cells. However, it is generally considered that HepG2 cells are insensitive to MSeA-induced death. In our current research, we found that the addition of PA increased the sensitivity of HepG2 cells to low-dose MSeA-induced apoptosis. The anticancer efficacy of the MSeA/PA combination was also demonstrated in a HepG2 xenograft model. Further experiments revealed that IRE1 inhibition significantly enhanced the PA-induced apoptosis, indicating the prosurvival function of IRE1 in PA treatment of HepG2 cells. The combination of PA and MSeA attenuated the IRE1 pathway and increased the expressions of phospha-eIF2α and GADD153/C/EBP homologous protein (CHOP), contributing to the PA/MSeA combination-induced mitochondria-dependent apoptosis in HepG2 cells. In addition, PA downregulated the expression of the glucose transporter GLUT1 and restricted glucose metabolism, thus promoting the apoptosis of tumor cells. Considering the lipotoxicity of PA, L02 human normal hepatocytes were used to evaluate the effect of MSeA on the lipotoxicity caused by PA. Interestingly, MSeA prevented PA-induced lipotoxicity in L02 cells. Our findings provided evidence that PA may be a promising and excellent sensitizer for improving the anticancer effect of MSeA in hepatoma chemotherapy.

INTRODUCTION

Hepatocellular carcinoma (HCC) has high morbidity and mortality rates, and there is currently no clear treatment method. Lipids are basic components and energy sources of cells, and changes in lipid composition are increasingly believed to be closely related to the occurrence of cancer. Palmitic acid (PA), a long-chain saturated fatty acid, is the most common saturated fatty acid in dietary fats. For example, in peanut oil, PA accounts for about 13% of the total fatty acid, 65% in butter, 42% in lard, 15% in soybeans, and so on. In addition, PA is the most common saturated fatty acid in our body, accounting for about 65% of the human saturated fatty acids. Although some studies have shown that PA has potential tumorigenic properties, there are also reports that PA decreases the cell membrane fluidity of hepatocellular carcinoma cells and restricts glucose metabolism. Moreover, PA downregulates the expression levels of mTOR and STAT3, reduces cancer cell proliferation, impairs cell invasiveness, and inhibits tumor growth in LM3 xenograft mouse models. In breast cancer, PA induces a functionally different transcription program, which reduces the expressions of HER2 and HER3. Additionally, PA plays an important role in the secretion of exosomes from cancer cells. Selenium is an essential trace element for the human body, and it plays an indispensable role in organisms, such as anticancer, immune regulation, detoxification, and antioxidation. Lack of selenium can cause a series of diseases. Methylseleninic acid (MSeA) is an important organoselenium derivative, which generates methylselenol through its spontaneous reaction with free thiols to exert anticancer effects. Because the activity of MSeA does not depend on the expression of lyses, such as methionine γ-lyase, it may be a more effective and promising antitumor drug than other organoselenium compounds. Some experimental and clinical research data indicate that low selenium intake is a related risk factor for primary liver cancer (PLC), and the liver is
particularly sensitive to the supply of selenium.\textsuperscript{8,9} However, high selenium intake can lead to diabetes and cardiovascular diseases.\textsuperscript{10−12} Although studies have confirmed that MSeA can induce apoptosis in HepG2 human liver cancer cells, its dosage seems to have potential health risks.\textsuperscript{13}

The endoplasmic reticulum (ER) is an important subcellular organelle, which plays a vital role in the process of protein synthesis, folding and maturation.\textsuperscript{14,15} For ER-mediated protein folding, the unfolded protein response (UPR) can maintain a homeostatic balance between the demand and capacity of mammalian cells.\textsuperscript{13−19} If the UPR fails to manage misfolded and unfolded proteins, the cellular apoptosis pathways are triggered.\textsuperscript{1,19,20} ER stress often induces cell apoptosis via CCAAT/enhancer-binding protein homologous protein (CHOP), which induces caspase activation through genes such as Bim\textsuperscript{21} and DR5.\textsuperscript{22−25} Lipotoxicity can induce cell apoptosis through a variety of mechanisms, including ER stress.\textsuperscript{26} Previous studies have shown that in PA-induced ER stress, the activation of JNK and the upregulation of CHOP are downstream events.\textsuperscript{27} MSeA has also been shown to cause overall redox reactions to modify proteins. These changes are intracellular stress caused by unfolded or misfolded proteins. Recent research has provided strong evidence to support the important role of ER stress in the anticancer effect of selenium.\textsuperscript{28} In PC3 cells, MSeA induces the hallmark signals of ER stress, including upregulation of phosphorylated PERK, phosphorylated eIF2\textalpha, and glucose-related proteins Bip and GRP94. Moreover, CHOP/GADD153 may be a key transcription factor in the process of MSeA-induced apoptosis.\textsuperscript{28}

Figure 1. Combined use of MSeA and PA enhanced the cytotoxicity in HepG2 liver cancer cells, and MSeA inhibited PA-induced apoptosis in L02 human normal liver cells. The cells were treated with the specified concentration of MSeA and/or PA for 24 h, and then the cells were collected for viability analysis. In the cell migration experiment, a sterile microtube tip was used to draw a line on the monolayer of cells to form a linear scratch, and then the effect of the drug on cell migration was observed. (A) Combination effect of PA/MSeA on HepG2 cells measured by crystal violet staining. (B) Promotive effect of PA on MSeA-induced apoptosis assessed by Annexin V/PI in HepG2 cells. (C) Inhibitory effect of MSeA on the total number of L02 cells caused by PA examined by crystal violet staining. (D) Protective effect of MSeA on PA-induced apoptosis in L02 examined by Annexin V/PI staining. (E) Representative microscopy images showed the reduction of HepG2 cell migration under MSeA and/or PA treatment. (F) Analysis of the experimental wound closure.
In our study, PA enhanced the sensitivity of HepG2 cells to low-dose MSeA in vitro and in vivo. In this process, IRE1 played a prosurvival role in PA-induced cell death. MSeA inhibited PA-induced p-IRE1α and further enhanced p-eIF2α and CHOP levels, which contributed to the PA/MSeA combination-induced mitochondria-dependent apoptosis in HepG2 cells.

**RESULTS**

**Combination of MSeA and PA Increases the Anticancer Effect of MSeA In Vitro and In Vivo.** First, we used crystal violet staining to detect the changes in cell viability induced by MSeA in the presence or absence of PA in HepG2 cells. Exposure to 2 μM MSeA alone did not cause a significant change in the cell survival rate, while the combination of MSeA and PA dramatically increased cytotoxicity (Figure 1A). Next, we used Annexin V/PI staining to further prove this effect. As shown in Figure 1B, the MSeA/PA combination significantly enhanced cell death compared with the effect of MSeA and PA alone. Taking into account the lipotoxicity of PA, we used L02 human normal hepatocytes to evaluate the changes in cell viability. As shown in Figure 1C, exposure to PA caused a significant decrease in cell viability, while MSeA greatly ameliorated the inhibition effect of PA on cell viability. Then, we used Annexin V/PI staining to further detect the influence on L02 cells. As shown in Figure 1D, PA induced an increase in apoptosis, which was significantly reduced after MSeA treatment.

Furthermore, we performed the wound-healing experiment to assess the effect of MSeA/PA treatment on the migration characteristics in HepG2 cells. As shown in Figure 1E,F, the MSeA/PA combination effectively inhibited the migration of HepG2 cells.

To further verify the synergy of MSeA/PA in vivo, we established the HepG2 xenograft mouse model. When the average tumor volume reached about 100−120 mm³, the HepG2 tumor-bearing mice were given MSeA and/or PA, respectively. The dose of MSeA was 2 mg/kg/day, and the dose of PA was 10.26 mg/kg/day. As shown in Figure 2A, the drug treatments did not cause a significant decrease in the weight of the mice. Moreover, the dose of each agent alone slightly reduced the size and weight of the tumor, while the MSeA/PA combination further inhibited the tumor growth (Figure 2B−D). These above-mentioned data indicate that the combination of MSeA and PA exerted better anticancer effects both in vitro and in vivo. Additionally, MSeA can protect against PA-induced cytotoxicity in L02 human normal liver cells and will not have greater toxic effects on normal liver cells.

**IRE1 Plays an Anti-Apoptotic Role in MSeA/PA Combination-Induced Cell Death.** Previous studies have demonstrated that the kinases, PERK and IRE1, alleviate ER stress by orchestrating the UPR. Under irresolvable ER stress, PERK activity persists, whereas IRE1 paradoxically attenuates, eventually promoting cell death. In this experiment, we measured the expression changes in the key ER stress markers, IRE1α and eIF2α. Western blot analysis demonstrated that the MSeA/PA combination treatment increased the phosphorylation of eIF2α but decreased the phosphorylation of IRE1α (Figure 3A), indicating that some coordination between two branches of the UPR determines
the apoptotic cell fate. The transcription factor, CHOP, is a key mediator of ER stress-induced apoptosis, and excessive or abnormal ER stress can lead to the activation of CHOP and ultimately cause cell death. We found that MSeA/PA also caused a significant upregulation of CHOP. Additionally, the expression of Bip increased. To determine the essential role of IRE1 in MSeA/PA-induced apoptosis in HepG2 cells, we used 4μ8C, an IRE1 inhibitor, to evaluate apoptosis induced by PA. We found that the inhibition of IRE1 by 4μ8C greatly increased PA-induced apoptosis (Figure 3C), as well as the expression of CHOP (Figure 3D). Furthermore, MSeA/PA inhibited the anti-apoptotic effect of IRE1 in the xenograft mouse model (Figure 3E). It was noteworthy that p-IRE1α was also inhibited by MSeA/PA in L02 (Figure 3B), suggesting that the IRE1 signaling pathway might play a distinct role in different cell types and different periods of intracellular stress. These results support the key role of ER stress, especially IRE1 inactivation in MSeA/PA-induced CHOP upregulation and cell death.

**PA Reduces the Expression of GLUT1 in the Combined Anticancer Effect of MSeA and Limits Glucose Metabolism.** Cancer cells have unique metabolic preferences, and they may upregulate the expression of glucose-related proteins or activate related transporters to increase glucose uptake. We tested the effects of MSeA and PA treatment on the expression of the glucose transporter GLUT1. Western blot analysis confirmed that MSeA did not change the expression of GLUT1, while the addition of PA reduced the level of GLUT1 in HepG2 cells, whether in cell membrane protein or total cell protein (Figure 4). Previous studies have shown that PA does not affect the content of GLUT1 in LM3 cells, while with the addition of PA, GLUT4 decreases in a dose-dependent manner. This may be because there are different results in different cells. In summary, we...
concluded that PA may limit glucose metabolism by downregulating GLUT1, making HepG2 more sensitive to MSeA-induced apoptosis.

**Cotreatment with MSeA and PA Induces Mitochondria-Dependent Apoptosis in HepG2 Cells.** The activation of the mitochondrial pathway plays a key role in PA-mediated apoptosis.31 The mitochondrial pathway is a major apoptosis signaling pathway, and one of its characteristics is the destruction of the mitochondrial membrane potential (MMP).31,32 We first studied the effect of MSeA on PA-mediated MMP destruction. As shown in Figure 5A, MSeA/PA cotreatment resulted in a significant increase in the disruption of MMP. We further examined the Bcl-2 family proteins under MSeA and/or PA exposure. As shown in Figure 5B, MSeA/PA significantly increased the expression of Bim. In addition, the increased cleavage of PARP and the down-regulation of full-length caspase-9 also proved the above results (Figure 5C). These data indicated that MSeA/PA induced mitochondria-dependent apoptosis in HepG2 cells.

**DISCUSSION**

PA is the most common saturated long-chain fatty acid in the human diet, and it acts as a signaling molecule to regulate various diseases at the molecular level.1 There are reports that the intake of saturated fatty acids, such as PA, can cause diseases related to lipotoxicity, and even cancer. However, in fact, owing to different metabolic reprogramming processes, the carcinogenic effects of PA on different cell types are distinct. As mentioned earlier, PA has been reported to reduce the proliferation and metastatic invasion in hepatocellular carcinoma. Furthermore, the addition of PA can reduce the cell membrane fluidity and limit glucose metabolism.1,3 In breast cancer cells, PA causes cell cycle delay in G2-phase and leads to CHOP-dependent apoptosis.4 Moreover, PA addition increases the sensitivity of HER2-neutral and positive breast cancer cells to trastuzumab treatment.4 Recently, researchers have found that PA reduced the secretion of exosomes in PC3 human prostate cancer cells in a concentration-dependent manner.5 In this study, we discovered that PA addition increased the sensitivity of HepG2 cells to low-dose MSeA-induced apoptosis (Figure 1B). Moreover, the combined effect of MSeA/PA was also demonstrated in the HepG2 xenograft model (Figure 2).

It has been well established that apoptosis is the main underlying mechanism of the anticancer activity of selenium. Our finding revealed that cotreating PA with MSeA induced...
apoptosis via the mitochondrial pathway (Figure 5). Mitochondria is the very important organelle that regulates cell metabolism, and its dysfunction is closely related to the occurrence and progression of various metabolic diseases. Tumor cells need to sense and adapt through metabolic reprogramming to survive, and mitochondria, as the core of cell energy metabolism regulation, is closely related to the metabolic adaptation of liver cancer cells. We found that PA may limit glucose metabolism by downregulating GLUT1, making HepG2 more sensitive to MSeA-induced apoptosis.

Studies have shown the role of ER stress in selenium-mediated apoptosis. Researchers have found that MSeA induced the production of ER stress markers in PC3 cells, such as p-PERK, p-eIF2α, and the apoptosis-related molecule CHOP. Additionally, in the process of MSeA-induced apoptosis, CHOP may be an important transcription factor. However, it has been shown that HepG2 cells are insensitive to low-dose MSeA. One reason for this may be that low-dose MSeA cannot induce CHOP expression in HepG2 cells (Figure 3A). In our study, the pro-apoptotic PERK signaling pathway was further enhanced by the addition of both MSeA and PA, contributing to CHOP-dependent cell death (Figure 3A). The UPR has contradictory results in different situations, including rebuilding cell homeostasis and promoting cell apoptosis. The UPR has three branches, of which IRE1 is the most conservative stress sensor among the three branches. IRE1 exerted both pro- and anti-apoptotic effects depending on the stress conditions. We found that MSeA reduced the PA-induced p-IRE1 level in HepG2 cells (Figure 3A) and in a xenograft mouse model (Figure 3E), suggesting its anti-apoptotic role in MSeA/PA hepatoma chemotherapy. Using IRE1 inhibitor in PA-treated HepG2 cells, we further verified the anti-apoptotic role of IRE1 under such conditions (Figure 3C,D). This also showed that in the combined anticancer effect of MSeA and PA, MSeA seems to act as an IRE1 inhibitor.

Finally, considering the potential lipotoxicity of PA, we performed a similar experiment in L02 human normal liver cells. Interestingly, we found that MSeA treatment protected against PA-induced cell death in L02 cells. Due to the decrease in intracellular stress, the phosphorylation levels of eIF2α and CHOP decreased. Surprisingly, the expression of p-IRE1α was downregulated as well, suggesting its pro-apoptotic effect in L02 cells. The IRE1 and PERK signaling pathways play the same role, and the addition of MSeA relieves the intracellular stress and protects the cells from PA lipotoxicity in L02 cells. The same experiment was also verified in human liver cancer cell SMMC-7721 (not shown in the data). Although the addition of 200 μM PA significantly decreased cell viability, and there was no statistically significant difference compared with the MSeA and PA combined treatment group. The addition of IRE1 inhibitor 4μ8C caused a slight decrease in cell viability, but the results were not statistically different. From The Human Protein Atlas database (https://www.proteinatlas.org/), we know that the expression levels of IRE1 in HepG2 are relatively high in many liver cancer cells, so we speculate that inhibiting IRE1 has a greater impact on the state of HepG2, which needs further research to prove. In summary, our results confirmed that IRE1 played a prosurvival role in PA-induced cell death. MSeA inhibited PA-induced p-IRE1α and further enhanced the p-eIF2α and CHOP levels, which contributed to the combination treatment-induced mitochondria-dependent apoptosis in HepG2 cells. Taken together, our study revealed that PA may be a promising sensitizer for improving the anticancer efficacy of MSeA in hepatoma chemotherapy.

**MATERIALS AND METHODS**

**Chemicals and Reagents.** Methylseleninic acid (MSeA, purity >95%) and palmitic acid (PA) (P0500) were purchased from Sigma-Aldrich (St. Louis, MO). IRE1α inhibitor 4μ8C was purchased from MCE (Shanghai, China). Primary antibodies specific for phospho-eIF2α (3398), c-PARP (9546), Bip (3183), Bim (2933), and caspase-9 (9502) were purchased from Cell Signaling Technology (Beverly, MA). Phospho-IRE1α (ab48187) was purchased from Abcam (Cambridge, MA). CHOP (15204-1-AP) was purchased from Protein Tech (Rosemont, IL). Secondary antibodies specific for rabbit and mouse immunoglobulins were purchased from MBL International Corporation (Woburn, MA).

**Cell Culture and Treatment.** Cells were maintained in a humidified incubator at 37 °C and 5% CO2. The cell lines HepG2 and L02 were obtained from the American Type Culture Collection (ATCC). HepG2 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) without antibiotics. L02 cells were cultured in RPMI-1640 Medium supplemented with 10% FBS without antibiotics. When the degree of cell fusion reached a suitable range, the medium was changed and then treated with the corresponding reagents.

**Crystal Violet Staining.** The crystal violet staining was used to evaluate the effect of PA and/or MSeA on cell viability. Cells were exposed to MSeA and/or PA for 24 h. After the treatments, the medium was removed and the cells were fixed in 1% glutaraldehyde solution in phosphate-buffered saline (PBS) for 15 min. The fixed cells were stained with 0.02% crystal violet for 30 min. After washing with PBS, the stained cells were dissolved with 70% ethanol. The absorbance at 570 nm with the reference filter of 405 nm was measured with a microplate reader (Thermo).

**Apoptosis Evaluation.** Apoptosis of cells was determined by Annexin V/PI double staining of externalized phosphatidylserine (PS) by flow cytometry using a kit from MBL International Corporation (Woburn, MA).

**Western Blotting.** The samples were lysed with ice-cold radioimmunoprecipitation assay (RIPA) buffer. An equal amount of the sample protein was loaded onto the gel. After separation by electrophoresis, the proteins were transferred to a nitrocellulose (NC) membrane. The membrane was incubated with the primary antibody and then recognized with the corresponding secondary antibody. Then, the immunoreacted bands were obtained using an X-ray film. Western blot images were quantitatively analyzed using ImageJ software.

**Cell Membrane Protein Extraction.** A membrane protein extraction kit (Beyotime Biotechnology, P0033) was used to extract cell membrane proteins, and the extracted proteins were used for the next electrophoresis.

**Wound-Healing Experiment.** Cells were seeded in a 6-well culture plate at a density of 5 × 10^4 cells/well until they grew to 90% confluence. Use a sterile 200 μL micropipette tip to create a linear scratch in the monolayer of cells. The cells were washed three times with PBS to remove the streaked cells and then incubated in a medium without fetal bovine serum or a serum-free medium containing drugs. Cell migration to the damaged area was observed at 12 and 24 h.
Mitochondrial Membrane Potential Measurement. Mitochondrial permeability transition (MPT) was determined using a JC-1 kit (M8650) from Solarbio Life Science (Beijing, China). After it was stained, flow cytometry was used to examine the changes of MPT.

Animals and Treatments. The animal care and experimental protocols in this study were approved by the Animal Care and Use Committee of China Agricultural University. After 7 days of acclimatization, all mice were randomly divided into four groups with three mice in each group. To establish tumor xenografts, HepG2 cells (∼2 × 10⁶) were mixed with 50% Matrigel (Corning, MA) and injected subcutaneously into the right flank of 6–8 week-old male BALB/c athymic nude mice (Charles River Laboratories, Beijing, China). Tumors were measured with a caliper, and the tumor volume was calculated according to the following formula: \( V = \frac{1}{2}w_1 \times w_2 \times w_3 \), where \( w_1 \) is the maximum diameter of the tumor and \( w_2 \) is the minimum diameter of the tumor. The body weight and tumor volume were evaluated every other day. When the tumor volume was up to about 100–120 mm³, continuous intragastric administration of PA (10.26 mg/kg/day), MSeA (2 mg/kg/day), and their combination medication were given for 20 days. At the end of the experiment, all animals were euthanized. Tumor tissues were collected and stored at −80°C, and a portion of tumors from control and treated animals were used to prepare tumor lysates for further analysis.

Statistical Analysis. All experiments have been carried out at least three times, and the corresponding data are given. All data are expressed as mean ± standard deviation (SD), and two-sided Student’s t test was used for normally distributed variables (*P < 0.05; **P < 0.01; ***P < 0.001). Multiple comparisons between groups were made by one-way analysis of variance (ANOVA) with Tukey’s post hoc test. Statistical significance was defined as P < 0.05 (*P < 0.05; **P < 0.01).

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Notes
The authors declare no competing financial interest.

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ABBREVIATIONS USED

PA, palmitic acid; MSeA, methylseleninic acid; CHOP, GADD153/C/EBP homologous protein; HCC, hepatocellular carcinoma; PLC, primary liver cancer; PARP, poly(ADP-ribose) polymerase; ER, endoplasmic reticulum; UPR, unfolded protein response; MPT, mitochondrial permeability transition; MMP, mitochondrial membrane potential

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