Conjugated Linoleic Acids Inhibit Hypoxia Inducible Factor-1 \( \alpha \) Stabilization under Hypoxic Condition in Human Hepatocellular Carcinoma Cells

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Abstract: The anti-cancer effects of various fatty acids are drawing a lot of attention. To determine whether different fatty acids affect the hypoxic response of liver cancer cells, we examined the effects of various fatty acids on the stabilization of the hypoxia-inducible factor (HIF)-1\( \alpha \) protein in the HepG2 human hepatocellular carcinoma (HCC) cell line under condition containing 1% O\(_2\). Of the fatty acids examined, only 9\( \text{cis} \), 11\( \text{trans} \) (c9, t11)-conjugated linoleic acid (CLA) and 10\( \text{trans} \), 12\( \text{cis} \) (t10, c12)-CLA inhibited hypoxia-induced HIF-1\( \alpha \) stabilization. In addition, HIF-1\( \alpha \) prolyl hydroxylase or proteasome inhibition abrogated the effects of c9, t11- and t10, c12-CLA. Moreover, c9, t11- and t10, c12-CLA significantly inhibited cell proliferation and induced apoptotic cell death under hypoxia. This is the first study showing that c9, t11- and t10, c12-CLA inhibit the hypoxic response in HCC cells.

Key words: conjugated linoleic acid, hypoxia inducible factor, apoptosis, hepatocellular carcinoma

1 INTRODUCTION

Accumulating data strongly suggest that various fatty acids exert anti-cancer effects that can be attributed to their ability to induce apoptotic cell death, regulate the cell cycle, manipulate eicosanoid production, etc.[1-3]. Although such anti-cancer effects of fatty acids have been proven in animal models, detailed mechanisms still remain to be elucidated. In solid tumors, it is well known that hypoxic regions occur inside the tumors because of lack of O\(_2\) supply from the surrounding blood vessels, and the adaptive response to hypoxia occurs in the tumor cells for survival and proliferation. Hypoxia-inducible factor (HIF)-1, a heterodimeric transcription factor composed of two subunits known as HIF-1\( \alpha \) and HIF-1\( \beta \), regulates cellular homeostasis in response to the cellular O\(_2\) concentrations. Under normoxia (20% O\(_2\)), HIF-1\( \alpha \) is constitutively expressed, while prolines 402 and 546 in the HIF-1\( \alpha \) protein are easily hydroxylated, leading to HIF-1\( \alpha \) ubiquitination and proteasomal degradation. On the other hand, under hypoxic conditions, HIF-1\( \alpha \) protein is stable, and it translocates into the nucleus to complex with HIF-1\( \beta \). HIF-1\( \alpha \) prolyl hydroxylase (PHD) is an oxygen-sensitive enzyme and is inactivated under normoxia. HIF-1 regulates the expression of over 70 genes involved in cell survival, proliferation, glycolysis, and angiogenesis, and most of these responses to hypoxia are beneficial for tumor development[4,5]. Therefore, inhibition of HIF-1\( \alpha \) stabilization is an attractive target that induces a pleiotropic phenotype unfavorable for cancer growth[6]. Several studies have shown that high levels of HIF-1\( \alpha \) are expressed in hepatocellular carcinoma (HCC), indicating that the hypoxic response plays an important role in HCC progression[7-10]. Here, our aim was to investigate the effects of various fatty acids on the HIF-1\( \alpha \) levels under hypoxia in order to determine whether fatty acids can attenuate the hypoxic response in the HCC cells.

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2 EXPERIMENTAL PROCEDURES

2.1 Materials
All fatty acids used in this study (palmitic acid, stearic acid, oleic acid, linoleic acid, c9, t11-conjugated linoleic acid (CLA), t10, c12-CLA, α-linolenic acid, γ-linolenic acid, arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid) were purchased from Cayman Chemical Company (Ann Arbor, MI). c9, t11-CLA and t10, c12-CLA are referred to in the figures as CLA1, and CLA2, respectively. Dimethyloxallyl glycine (DMOG), YC-1 and MG132 (Cayman Chemical Company, Ann Arbor, MI), were used as PHD, HIF-1 and 26S proteasome inhibitors, respectively.

2.2 Cells and cell culture
A human HCC line, HepG2, was purchased from the Japan Cancer Research Resources Bank (Osaka, Japan). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma, St. Louis MO) containing 100 units/ml penicillin G and 100 μg/ml streptomycin supplemented with 10% fetal bovine serum (FBS) at 5.0 × 10^5 cells/90-mm dish. The number of viable attached cells was measured after trypsinization (0.2% trypsin with 0.025% EDTA in phosphate-buffered saline (PBS)) by using the 0.2% trypan blue exclusion method. Before the actual experiment, cells were plated and cultured for 1 day in DMEM supplemented with 10% FBS before being used for testing samples, in order to negate any possible effects of fatty acids on cell attachment. Then, cells were treated with 50 μM fatty acids for 24 h before the hypoxic exposure. Environmental hypoxia (1%) was achieved by continuously flushing an airtight humidified chamber with a gas mixture containing 5% CO₂ and 94% N₂.

2.3 Western blot analysis
Proteins (HIF-1α, β-actin, and histone H1) were detected using western blot analysis. After the culture period, cells were washed with ice-cold PBS, and then lysed in 1% Triton-X-50 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, 50 mM NaF, and 30 mM Na₄P₂O₇. Debris was removed by centrifugation at 12000 × g at 4°C for 20 min, and the resultant supernatant was used. Nuclear and Cytoplasmic Extraction Reagents (NE-PER, Pierce, Rockford, IL) was used to collect nuclear protein fractions. After determination of protein content, 10-μg protein samples were separated by electrophoresis on SDS-polyacrylamide gels, and then transferred onto PVDF membranes (GE Healthcare, Buckingham, UK). After blocking in 5% non-fat dried milk for 1 h at 37°C, the membranes were incubated with rabbit anti-HIF-1α antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-β-actin (Sigma, MO), or anti-histone H1 (Acris Antibodies, Herford, Germany) overnight at 4°C. Finally, the membranes were treated with peroxidase-conjugate goat anti-rabbit IgG (Santa Cruz Biotechnology) for 60 min at 25°C. After each antibody-binding reaction, the membranes were washed with Tris-buffered saline (pH 7.4) containing 0.1% Tween-20. Detection of each protein was performed using an ECL Prime kit (GE Healthcare). Band intensities were quantified using Image J software, and the figures show the representative blot patterns and the mean values for 3 independent experiments.

2.4 Cell cycle and apoptosis analysis
For cell cycle analysis, cells were fixed in ice-cold methanol for 30 min. Next, cells were treated with 10 μg/ml propidium iodide and 10 μg/ml RNase. Analysis of cell cycle and detection of the sub-G1 population were performed using a flow cytometer Cell Lab Quanta SC MPL (Beckman Coulter, Inc., Fullerton, CA) equipped with MultiCycle software (San Diego, CA).

2.5 Statistical analysis
Data were analyzed using a Student’s t-test to evaluate differences among groups. Values marked with asterisk (⁎) are significantly different from the control values at p < 0.05* or 0.01**.

3 RESULTS AND DISCUSSION
To examine the hypoxic response of HepG2 cells, cells were cultured under conditions containing 20% or 1% O₂, and HIF-1α protein levels were analyzed by western blot analysis. Apparent accumulation of HIF-1α in HepG2 cells was already seen at 1 h after exposure, and reached a plateau approximately 4 h later (data not shown). Therefore, cells were exposed to hypoxic conditions for 4 h in the subsequent experiments for evaluation of HIF-1α accumulation. First, we examined the effects of 11 saturated, monounsaturated, and polyunsaturated fatty acids at a concentration of 50 μM on hypoxia-induced HIF-1α stabilization (Fig. 1A). Here, YC-1 is positive control for the inhibition of HIF-1α stabilization. Preliminary studies revealed that these fatty acids at 50 μM did not have any cytotoxic effects on HepG2 cells, at least at the end of 4 h of hypoxic exposure. When cells were pretreated with fatty acids for 24 h before hypoxia treatment, c9, t11- and t10, c12-CLA significantly inhibited hypoxia induced HIF-1α accumulation. Some other fatty acids tended to inhibit HIF-1α accumulation albeit moderately. In addition, c9, t11- and t10, c12-CLA, but not LA, decreased the nuclear HIF-1α levels (Fig. 1B). As previously reported, the electrophoretic migration pattern of HIF-1α of HepG2 cells was diffuse because of its post-translational modification [10].

How do CLA inhibit HIF-1α accumulation under hypoxia? As cellular HIF-1α levels are largely maintained under the regulation of ubiquitin-proteasomal degradation, we examined the effects of c9, t11- and t10, c12-CLA on HIF-1α degradation under hypoxia. When the cells were
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treated with MG132 or DMOG, which are inhibitors for 26S proteasome and PHD, they induced HIF-1α accumulation under normoxia and hypoxia, suggesting proteasomal degradation significantly affects on the HIF-1α expression under normoxia and hypoxia (Figs. 2A, 2B). Here again, when cells were treated with c9, t11- and t10, c12-CLA, HIF-1α expression was suppressed under hypoxia (Figs. 2A, 2B). On the other hand, Figs. 2A and 2B showed that MG132 and DMOG completely cancelled this HIF-1α suppressive effect of c9, t11- and t10, c12-CLA under hypoxia.

To determine the anti-proliferative effects of CLA on HepG2 cells under hypoxia, cells were pretreated with LA, c9, t11- and t10, c12-CLA for 24 h, and then exposed to hypoxic conditions for 48 h. Viable cell numbers and cell viability were then analyzed. Cell proliferation was strongly inhibited under hypoxia compared with normoxia (Fig. 3A). Under normoxia, although LA and c9, t11- and t10, c12-CLA slightly inhibited the proliferation of HepG2 cells, these differences were not significant. Moreover, t10, c12-CLA significantly increased the subG1 population under hypoxia (Fig. 3B).

It is well known that several food components have the ability to prevent the activation of HIF-1α-dependent pathways under hypoxia, and that this action is expected to contribute to the observed anti-cancer effects of these food components. Several fatty acids have potent anti-cancer effects in experimental animal models, and some of them clearly show anti-angiogenic activity. Although angiogenesis is a cellular defense against the hypoxic response when the tumor tissue is exposed to hypoxia, systemic approaches for the evaluation of fatty acids with the hypoxic response have not been performed. Here, we found that CLA has the ability to prevent HIF-1α stabilization under hypoxia. As far as we know, this is the first
study showing the anti-hypoxic effects of CLA.

CLA is a generic term for the geometric and positional isomers of octadecadienoic acids with conjugated structures. Among them, the c9, t11 and t10, c12 isomers perform various physiological functions, either individually or in association with each other\(^{14, 15}\). Anti-tumor functions such as anti-carcinogenic and tumor cell-killing activities are paradigms of the beneficial physiological effects of CLA. As an underlying mechanism of the anti-cancer effects of CLA \textit{in vivo}, CLA is known to inhibit vascular endothelial growth factor (VEGF) production and angiogenesis\(^{16, 17}\), but the detailed mechanism for anti-angiogenesis is still unknown. As VEGF is one of representative target proteins of HIF-1, our present study in which c9, t11- and t10, c12-CLA inhibited HIF-1α stabilization provides a novel aspect for elucidating the anti-angiogenesis mechanism of CLA. Moreover, the present data showed that the inhibition of PHD or 26S proteasome activities abrogated the effect of c9, t11- and t10, c12-CLA, suggesting that HIF-1 degradative activity was sustained in the CLA-treated cells. Thus, PHD activity may be maintained under hypoxia with c9, t11- and t10, c12-CLA treatment. Although cells were pretreated with CLAs for 24 h before hypoxic treatment in this experiment, our preliminary study showed that 1 h pretreatment with CLA was not effective for the suppression of HIF-1α (data not shown), suggesting the necessity of 24 h pretreatment. In previous experiment using isolated mitochondria, it was shown that pretreatment with 50 μM c9, t11- and t10, c12-CLA equally inhibited ADP-stimulated respiration on palmitoleic acid\(^{18}\). Therefore, we speculated that these CLAs inhibited oxygen consumption of HepG2 cells and kept their intracellular oxygen level even in hypoxia that was enough to maintain proteasomal HIF-1α degradation activity.

Fig. 2 Inhibition of HIF-1α proline hydroxylase or 26S proteasome abrogate conjugated linoleic acid-induced HIF-1α stabilization in HepG2 cells.

Cells were inoculated at a density of 5.0 \times 10^4 cells/well and cultured in 10% FBS-DMEM for 24 h, and then treated with appropriate concentrations of 9c, 11t, 10t, 12c-CLA, or LA for 24 h. After that, cells were cultured under conditions containing 20% (normoxia) or 1% (hypoxia) O\(_2\) for 4 h. One hour prior to hypoxic treatment, cells were treated with 10 μM MG132 (A) or 500 μM DMOG (B). Numbers show the mean values of the quantified band intensities calculated as the ratio of HIF-1α to β-actin for 3 independent experiments. Data are shown as relative band intensity for the None with hypoxic exposure and summarized as the mean ± SE Asterisk mark(s) denote significant differences from hypoxia control at \(p < 0.05\). LA: linoleic acid, CLA1: c9, t11-conjugated linoleic acid (CLA), CLA2: t10, c12-CLA
c9, t11- and t10, c12-CLA have been reported to exert cell-killing activity in various tumor cell lines through the induction of the apoptotic cell death pathway in vitro and in vivo. In the case of HCC cells, we previously reported that t10, c12-CLA, but not 9c, 11t-CLA or LA, exert potent cytotoxic effects on dRLh-84 rat hepatoma by activating the apoptosis pathway. On the other hand, present data showed that c9, t11- and t10, c12-CLA inhibit cell proliferation and induce apoptosis. When cells are rendered hypoxic, they undergo cell cycle arrest and apoptosis. Actually, as shown in Fig. 3, the proliferation of HepG2 cells was inhibited under hypoxia. On the contrary, apparent apoptosis was not detected in this experiment. It has been reported that activation of the apoptotic pathway under hypoxia is likely to occur during the early stages of tumor development, but some selected cancer cells escape from hypoxia-induced apoptosis. Therefore, it is considered that HepG2 showed substantial resistance to hypoxia-induced apoptosis. As shown in Fig. 3B, because t10, c12-CLA significantly induced apoptosis in the HepG2 cells, t10, c12-CLA may potentially eliminate apoptotic resistance to hypoxic stress in the HCC cells. Cancer cells in hypoxic state exist in an environment separated from adjacent vessels and are deficient in oxygen and nutrients supply. In such a situation, although it seems reasonable that CLAs could not reach the hypoxic area, our data reveal that pretreatment of HepG2 cells with CLAs under normoxic condition impairs their hypoxic response. Actually, as dietary CLA is known to be accumulated in the liver, CLA is expected to be incorporated into the cells in the normoxic area and to confer a unresponsiveness to hypoxia.

4 CONCLUSION
Here, as revealed by our data, c9, t11- and t10, c12-CLA inhibited HIF-1α stabilization and induced apoptosis in HepG2 cells under hypoxia. To our knowledge, although this is the first report of the inhibitory effects of c9, t11- and t10, c12-CLA on HIF-1α stabilization, further detailed mechanisms remain to be elucidated.

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