Apolipoprotein A-I Mimetic Peptides Inhibit Expression and Activity of Hypoxia-Inducible Factor-1 α in Human Ovarian Cancer Cell Lines and a Mouse Ovarian Cancer Model^[S]

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ABSTRACT

Our previous results demonstrated that the apolipoprotein A-I (apoA-I) mimetic peptides L-4F and L-5F inhibit vascular endothelial growth factor production and tumor angiogenesis. The present study was designed to test whether apoA-I mimetic peptides inhibit the expression and activity of hypoxia-inducible factor-1 α (HIF-1 α), which plays a critical role in the production of angiogenic factors and angiogenesis. Immunohistochemistry staining was used to examine the expression of HIF-1 α in tumor tissues. Immunoblotting, real-time polymerase chain reaction, immunofluorescence, and luciferase activity assays were used to determine the expression and activity of HIF-1 α in human ovarian cancer cell lines. Immunohistochemistry staining demonstrated that L-4F treatment dramatically decreased HIF-1 α expression in mouse ovarian tumor tissues. L-4F inhibited the expression and activity of HIF-1 α induced by

low oxygen concentration, cobalt chloride (CoCl₂, a hypoxia-mimic compound), lysophosphatidic acid, and insulin in two human ovarian cancer cell lines, OV2008 and CAOV-3. L-4F had no effect on the insulin-induced phosphorylation of Akt, but inhibited the activation of extracellular signal-regulated kinase and p70s6 kinase, leading to the inhibition of HIF-1 α synthesis. Pretreatment with L-4F dramatically accelerated the proteasome-dependent protein degradation of HIF-1 α in both insulin- and CoCl₂-treated cells. The inhibitory effect of L-4F on HIF-1 α expression is in part mediated by the reactive oxygen species-scavenging effect of L-4F. ApoA-I mimetic peptides inhibit the expression and activity of HIF-1 α in both in vivo and in vitro models, suggesting the inhibition of HIF-1 α may be a critical mechanism responsible for the suppression of tumor progression by apoA-I mimetic peptides.

Introduction

Because of the lack of tests to diagnose ovarian cancer at an early stage and the absence of effective therapeutic strategies, more than 70% of patients are diagnosed with late-

stage disease and a 5-year survival rate of only 50%. We reported previously that serum apolipoprotein A-I (apoA-I) levels are significantly decreased in patients with ovarian cancer, and apoA-I could be used as a biomarker for the detection of early-stage ovarian cancer (Kozak et al., 2003, 2005; Su et al., 2007). Our results demonstrated that the overexpression of apoA-I inhibits tumor growth and improves survival in a mouse ovarian cancer model (Su et al., 2010). We further showed that apoA-I mimetic peptides (18 amino acids in length compared with 243 amino acids for apoA-I) inhibited tumor growth similar to apoA-I overexpression in mouse models of ovarian cancer (Su et al., 2010).

ApoA-I mimetic peptides do not have sequence homology to apoA-I. However, these peptides have the capacity to form class A amphipathic helices similar to those found in apoA-I

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ABBREVIATIONS: apoA-I, apolipoprotein A-I; HIF-1, hypoxia-inducible factor-1; CoCl₂, cobalt chloride; LPA, lysophosphatidic acid; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor; CHX, cycloheximide; DAPI, 4,6-diamidino-2-phenylindole; DCFH-DA, dichlorofluorescein diacetate; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRE, hypoxia response element; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; RT, reverse transcription; sc-4F, scrambled 4F; SOD, superoxide dismutase; MG-132, *N*-(benzyloxycarbonyl)leucinylleucinylleucinal-Z-Leu-Leu-Leu-al.

and mimic lipid binding properties of apoA-I, producing antioxidant and anti-inflammatory effects (Navab et al., 2005, 2006; Shah and Chyu, 2005; Getz et al., 2009). Based on the number of hydrophobic phenylalanine (F) residues in the sequence, the peptides are named 2F, 3F, 4F, 5F, 6F, and 7F. To account for the balance between solubility in an aqueous environment and the ability to interact with lipids, we have used both 4F and 5F in cancer studies (Su et al., 2010). Our data showed that L-4F and L-5F (L standing for L amino acids) significantly inhibit tumor growth in a mouse ovarian cancer model (Su et al., 2010). Tumor angiogenesis plays a critical role in the growth and progression of solid tumors, including ovarian cancer (Folkman, 1971; Hanahan and Folkman, 1996; Carmeliet and Jain, 2000). Among the angiogenic factors, vascular endothelial growth factor (VEGF) is involved in every step of new vessel formation, including the proliferation, migration, invasion, tube formation of endothelial cells, and recruitment of various types of angiogenesis-associated cells, including VEGF receptor 1-positive cells and endothelial progenitor cells (Rafii et al., 2002; Adams and Alitalo, 2007; Ellis and Hicklin, 2008). More recently, we showed that the suppression of tumor growth is mediated, at least in part, by inhibition of the production of VEGF and subsequent tumor angiogenesis (Gao et al., 2011).

Expression and activity of hypoxia-inducible factor 1 (HIF-1) is crucial for the production of VEGF and other angiogenic factors in tumor tissues. HIF-1 is a heterodimeric transcription factor that consists of a constitutively expressed HIF-1 β and an inducible α -subunit, HIF-1 α . When tumor tissues overgrow, tumor cells located more than 100 μ m from vessels are under hypoxic conditions. Because of the oxygen-dependent nature of HIF-1 α degradation, low oxygen concentration leads to decreases of protein degradation, resulting in HIF-1 α accumulation. On the other hand, some hormones and growth factors, including insulin and lysophosphatidic acid (LPA), also promote protein accumulation of HIF-1 α by activating various signaling pathways under normoxic conditions (Cao et al., 2004; Lee et al., 2006, 2009). HIF-1 α binds to HIF-1 β , translocates into the nucleus, and contributes to tumorigenesis through the transcriptional activation of downstream genes, the protein products of which are required for angiogenesis (including VEGF and angiopoietins), glucose transport, and cell survival (Semenza, 2003; Pouyssegur and Mechta-Grigoriou, 2006; Pouyssegur et al., 2006). In this article, we examined the effect of L-4F and L-5F on the expression and activity of HIF-1 α in human ovarian cancer cell lines and mouse ovarian tumor tissues to delineate the mechanisms behind the antiangiogenic and antitumorogenic effects of apoA-I mimetic peptides.

Materials and Methods

Cells, Cell Culture, and Reagents. OV2008 cells were cultured in RPMI 1640 media with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μ g/ml), 1 $\times$ minimal essential medium nonessential amino acid solution (Invitrogen, Carlsbad, CA), and insulin (0.25 U/ml) (Invitrogen). CAOV-3 cells were cultured in complete media consisting of Dulbecco's modified Eagle's medium with high glucose and L-glutamine (2 mM), 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μ g/ml), and insulin (0.02 U/ml). To create hypoxic conditions, cells were transferred to a hypoxic chamber (model 3130; Thermo Fisher Scientific, Waltham, MA),

where they were maintained at 37°C in an atmosphere containing 5% CO₂, 1% O₂, and 94% N₂.

L-4F (the peptide Ac-D-W-F-K-A-F-Y-D-K-V-A-E-K-F-K-E-A-F-NH₂ synthesized from all L amino acids) was dissolved in water at 1 mg/ml (freshly prepared every time) and used between 1 and 10 μ g/ml. L-5F was synthesized by Peptisyntha Inc. (Torrance, CA), dissolved in ABCT buffer (50 mM ammonium bicarbonate, pH 7.0, containing 0.1 mg/ml Tween 20) at 1 mg/ml, and diluted to the required concentrations before use. Cobalt chloride (CoCl₂), insulin, cycloheximide (CHX), and *N*-(benzyloxycarbonyl)leucinylleucinylleucinal-Z-Leu-Leu-Leu-al (MG-132) were purchased from Sigma-Aldrich (St. Louis, MO). LPA (Avanti Polar Lipids, Alabaster, AL) in chloroform was dried as recommended by the manufacturer, dissolved in ethanol at a concentration of 20 mM as a stock solution, and diluted to the required concentrations in the corresponding cell culture media before use.

Quantitative Real-Time PCR. Total RNA was extracted from cells by using a PureLink RNA Mini Kit (Invitrogen). The quantity and quality of RNA were assessed by using a SmartSpec 3000 Spectrophotometer (Bio-Rad Laboratories, Hercules, CA). cDNA was synthesized by using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. PCRs were performed by using the CFX96 real-time PCR system (Applied Biosystems). The cycling conditions were as follows: 3 min at 95°C followed by 40 cycles of 95°C, 10 s; 60°C, 10 s; 72°C, 30 s followed by a final extension at 72°C for 10 min. Each 25- μ l reaction contained 0.4 μ g of cDNA, 12.5 μ l of SYBR Green qPCR SuperMix (Bio-Rad Laboratories), and 250 nM forward and reverse primers in nuclease-free water. Primers used were: HIF-1 α , 5'-TCC AGT TAC GTT CCT TCG ATC A-3' and 5'-TTT GAG GAC TTG CGC TTT CA-3'; VEGF, 5'-CGG CGA AGA GAA GAG ACA CA-3' and 5'-GGA GGA AGG TCA ACC ACT CA-3'; glucose transporter-1, 5'-CGG GCC AAG AGT GTG CTA AA-3' and 5'-TGA CGA TAC CGG AGC CAA TG-3'; aldolase-A, 5'-TGC TAC TAC CAG CAC CAT GC-3' and 5'-ATG CTC CCA GTG GAC TCA TC-3'; and GAPDH, 5'-GGA AGG TGA AGG TCG GAG TCA-3' and 5'-GTC ATT GAT GGC AAC AAT ATC CAC T-3'. The experiment was repeated once with triplicate measurements in each experiment.

Western Blot Analysis. Western blot analyses were performed as described previously (Gao et al., 2011). In brief, cell lysates were collected in a lysis buffer containing 0.1 M NaCl, 5 mM EDTA, 50 μ M sodium orthovanadate, 1% Triton X-100, and protease inhibitor tablet (Roche Diagnostics, Indianapolis, IN) in 50 mM Tris buffer, pH 7.5, loaded onto 4 to 12% Bis-Tris gel (Invitrogen), transferred to polyvinylidene difluoride membrane, and incubated with the appropriate antibodies. Anti-pThr²⁰²/Tyr²⁰⁴-Erk, anti-Erk, anti-pThr³⁸⁹-p70 S6 kinase, anti-p70 S6 kinase, anti-pSer⁴⁷³-Akt, and anti-Akt antibodies were purchased from Cell Signaling Technology (Danvers, MA); mouse anti-human HIF-1 α antibody was purchased from BD Pharmingen (San Diego, CA); rabbit anti-mouse HIF-1 α antibody was purchased from Abcam Inc. (Cambridge, MA); and anti-GAPDH antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Measurement of Cellular Reactive Oxygen Species. As described previously (Zhou et al., 2007; Lee et al., 2009), OV2008 cells were plated onto a glass slip (Thermo Fisher Scientific) in a 24-well plate at 4×10^4 cells per well, cultured overnight in normal cultured condition, starved in serum-free media overnight, and treated with L-4F (10 μ g/ml) for 1 h. Then, dichlorofluorescein diacetate (DCFH-DA, 10 μ M) and insulin (200 nM)/CoCl₂ (100 μ M) were added and incubated with the cells for an additional 0.5 h. The cells were washed twice with phosphate-buffered saline (PBS). The images were captured with a fluorescence microscope (Olympus IX70; Olympus, Tokyo, Japan).

Hypoxia Response Element Reporter Assay. In brief, OV2008 cells were plated at 2×10^5 cells per well in a six-well plate and grown in complete media overnight. Then, pGL3-Epo-hypoxia response element (HRE)-Luc plasmid was transfected into cells by

using Lipofectamine 2000 (Invitrogen). After 24 h, cells were starved overnight and subjected to L-4F treatment in the presence or absence of stimulators. A reporter assay system (Promega, Madison, WI) was used for the measurement of luciferase activity.

Immunofluorescence Staining of HIF-1 α . Immunofluorescence staining was performed as described previously (Lee et al., 2006). In brief, OV2008 cells were plated onto a glass slip (Thermo Fisher Scientific) in 24-well plates at 4×10^4 cells per well and grown in complete medium overnight. After starvation overnight, cells were subjected to L-4F treatment in the presence or absence of stimulators. Then, cells were fixed in 4% neutral buffered formaldehyde for 25 min at room temperature, permeabilized with 0.5% Triton X-100 in PBS for 10 min, and blocked with 10% normal goat serum, 1% bovine serum albumin, and 0.3 M glycine prepared in PBS for 1 h. Cells were incubated with mouse anti-HIF-1 α (1:200) overnight at 4°C and incubated with Alexa Fluor 568 goat anti-mouse IgG (Invitrogen) for 1 h. Finally, cells were covered with VectaMount solution containing DAPI (Vector Laboratories, Burlingame, CA), and images were captured with a fluorescence microscope (Olympus IX70).

In Vivo Tumor Model. Nine-week-old C57BL/6J female mice were given a 0.5-ml subcutaneous injection of 5×10^6 ID8 cells prepared as a single cell suspension in PBS mixed with an equal volume of cold Matrigel (BD Biosciences, San Jose, CA). After 2 weeks, mice started to receive scrambled 4F peptide (sc-4F) or L-4F (10 mg/kg) by subcutaneous injection at a site distant from the site where the ID8 cells were injected daily for 3 weeks. After 3 weeks, the mice were sacrificed for tumor collection and further analyses.

Immunohistochemistry Staining. Frozen tumor tissues were sectioned at a thickness of 5 μ m and fixed with cold acetone for 10 min at -20°C. The sections were blocked with 10% normal goat serum and 4% bovine serum albumin prepared in PBS for 3 h and immediately incubated with rabbit anti-mouse polyclonal HIF-1 α antibody (1:200) (Abcam Inc.) or rat anti-mouse monoclonal CD31 antibody (1:25) (Abcam Inc.) overnight at 4°C. The sections were

then incubated with corresponding biotinylated secondary antibodies (Vector Laboratories) for 30 min at room temperature followed by incubation with Vectastain ABC Elite reagents (Vector Laboratories) to visualize the staining. Finally, sections were lightly counterstained with hematoxylin, dehydrated, and coverslipped with VectaMount solution (Vector Laboratories).

Statistics. Data are shown as mean $\pm$ S.D. for each group. We performed statistical analyses by unpaired *t* test. Results for all tests were considered significant if *P* < 0.05.

Results

L-4F Inhibits HIF-1 α Expression and Angiogenesis

In Vivo. Our previous data showed that the apoA-I mimetic peptides L-4F and L-5F inhibited tumor growth and angiogenesis in an immunocompetent mouse model of ovarian cancer that uses the epithelial cancer cell line ID8 (Gao et al., 2011). Given the importance of HIF-1 α in the production of VEGF, a critical growth factor implicated in tumor angiogenesis, we first examined the effect of L-4F on HIF-1 α expression by using the same model. Immunohistochemistry staining showed that L-4F treatment decreased HIF-1 α expression in tumor tissues compared with a control peptide (sc-4F)-treated group (Fig. 1A). Consistent with our previous report (Gao et al., 2011), we observed a reduction in the number of vessels in L-4F-treated mice compared with the control group (Fig. 1A; Supplemental Fig. 1A).

L-4F and L-5F Inhibits HIF-1 α Expression in Cell Cultures. To examine whether L-4F inhibits HIF-1 α expression in cells under hypoxic conditions, low oxygen concentration (1% O₂) and a hypoxia mimetic chemical, CoCl₂, were used to induce HIF-1 α expression in a human ovarian cancer

Fig. 1. The ApoA-I mimetic peptide L-4F inhibits HIF-1 α expression in vivo and in vitro. **A**, an apoA-I mimetic peptide, L-4F, inhibits HIF-1 α expression and angiogenesis in vivo. Flank tumors were established in wild-type C57BL/6J mice as described under *Materials and Methods*. Two weeks after tumor growth, mice were treated with scrambled peptide (sc-4F) or L-4F (10 mg/kg s.c., daily injection) for 3 weeks. Frozen sections (5 μ m) from dissected tumors were subjected to hematoxylin and eosin (H&E) staining (left), HIF-1 α staining (center), and CD31 staining (right). Analysis was done from four randomly selected fields per slide (*n* = 4 mice per group). Representative figures are shown at 400 $\times$ magnification. Arrows indicate HIF-1 α -positive staining. **B**, pretreatment of L-4F inhibits CoCl₂- and insulin-induced HIF-1 α expression in human ovarian cancer cell lines. Cells were treated with vehicle or different concentrations of L-4F (1, 3, and 10 μ g/ml) for 1 h, and the indicated stimulators were added for another 4 h. Left, pretreatment of L-4F inhibits CoCl₂- and insulin-induced HIF-1 α expression in OV2008 cells. Right, pretreatment of L-4F inhibits CoCl₂- and insulin-induced HIF-1 α expression in CAOV-3 cells. **C** and **D**, L-4F decreases CoCl₂-induced (**C**) and insulin-induced (**D**) nuclear expression of HIF-1 α in OV2008 cells. Cells were immunostained with a mouse monoclonal anti-HIF-1 α primary antibody and a goat anti-mouse IgG labeled with Alexa Fluor 568 (red fluorescence) as the secondary antibody. DAPI was used to stain nuclei (blue). Images are shown at the original magnification of 200 $\times$. Dotted line and boxes show the area where the enlarged images originated. Representative photographs of two independent experiments with similar results are shown. The concentrations of stimulators used were: CoCl₂, 100 μ M, and insulin, 200 nM.

cell line, OV2008. Western blot analysis showed that L-4F dose-dependently suppressed hypoxia-induced HIF-1 α protein expression (Fig. 1B; Supplemental Fig. 1B). Similar results were observed when OV2008 cells were treated with insulin at 100 nM (data not shown) and 200 nM (Fig. 1B) and LPA at 20 μ M (Supplemental Fig. 1B).

To further confirm the inhibitory role of L-4F in HIF-1 α expression, two other human ovarian cancer cell lines, CAOV-3 and SKOV3, were studied. Consistent with the data for OV2008 cells, L-4F dose-dependently inhibited CoCl₂- and insulin-induced HIF-1 α expression in both CAOV-3 cells (Fig. 1A) and SKOV3 cells (data not shown).

To examine whether the inhibitory effect on HIF-1 α is specific to L-4F, another apoA-I mimetic peptide, L-5F, was used to treat OV2008 cells. Similar to L-4F treatment, L-5F dose-dependently inhibited low oxygen- and CoCl₂-stimulated HIF-1 α expression (Supplemental Fig. 1C).

As a transcription factor, HIF-1 α functions in nuclei and activates expression of downstream genes. Immunofluorescence staining was used to examine the effect of L-4F on the nuclear levels of HIF-1 α protein. CoCl₂ and insulin treatments greatly increased the accumulation of HIF-1 α in nuclei of OV2008 cells, and pretreatment of L-4F dramatically reversed these effects (Fig. 1, C and D).

Inhibition of HIF-1 α -Dependent Gene Transcription by L-4F. To determine whether L-4F inhibits HIF-1 α -driven gene transcription, OV2008 cells were transfected with a HRE containing luciferase reporter plasmid. L-4F treatment significantly inhibited CoCl₂- and insulin-mediated induction of luciferase activity (Fig. 2, A and C). Moreover, L-4F treatment abrogated CoCl₂- and insulin-induced increase in mRNA levels of HIF-1 α target genes including VEGF, glucose transporter 1, and aldolase-A (Fig. 2, B and D), suggesting that L-4F inhibits both HIF-1 α protein expression and activity.

Post-Treatment of L-4F Decreases HIF-1 α Protein Level and Activity in CoCl₂- and Insulin-Treated OV2008 Cells. Because HIF-1 α expression is elevated in advanced tumors that are presented clinically, we next examined whether L-4F given after hypoxia or growth factor stimulation inhibited HIF-1 α expression. OV2008 cells were stimulated first with CoCl₂ or insulin for 3 h (Supplemental Fig. 2) or 24 h (Fig. 3), and then treated with L-4F for various durations. Post-treatment of L-4F significantly decreased HIF-1 α expression in OV2008 cells (Fig. 3A; Supplemental Fig. 2A). Immunofluorescence analysis showed decreased nuclear expression of HIF-1 α by post-treatment of L-4F (Fig. 3B; Supplemental Fig. 2B). Moreover, down-regulation of HIF-1 α protein in the nucleus correlated with the inhibition

Fig. 2. HIF-1 α target gene expression is inhibited by L-4F in OV2008 cells. A, CoCl₂-stimulated HRE reporter gene transcription is inhibited by pretreatment of L-4F. OV2008 cells were transfected with pGL3-Epo-HRE-Luc plasmid and grown in complete growth media for 24 h. After an overnight starvation, cells were first treated with L-4F (10 μ g/ml) for 1 h and then treated with CoCl₂ (100 μ M) for an additional 6 h. Luciferase activity was determined as described under *Materials and Methods*. B, L-4F inhibits expression of HIF-1 α target genes in CoCl₂-treated cells. After serum starvation overnight, OV2008 cells were treated with L-4F (10 μ g/ml) for 1 h and then treated with CoCl₂ (100 μ M) for an additional 6 h. Total RNA was isolated, and the expression of VEGF, glucose transporter 1 (GLUT1), and aldolase-A (ALDO-A) mRNA levels were measured by real-time RT-PCR. GAPDH was used for normalization. C, insulin-stimulated HRE reporter gene transcription is inhibited by the pretreatment of L-4F. OV2008 cells were transfected with pGL3-Epo-HRE-Luc plasmid and grown in complete growth media for 24 h. After starvation overnight, cells were treated with L-4F (10 μ g/ml) for 1 h and then treated with insulin (200 nM) for an additional 16 h. Luciferase activity was determined as described under *Materials and Methods*. D, L-4F inhibits the expression of HIF-1 α target genes in insulin-treated cells. After serum starvation overnight, OV2008 cells were treated with L-4F (10 μ g/ml) for 1 h and then treated with insulin (200 nM) for an additional 16 h. Total RNA was isolated and the expression of VEGF, glucose transporter 1 (GLUT1), and aldolase-A (ALDO-A) mRNA levels were measured by real-time RT-PCR. GAPDH was used for normalization. #, $p < 0.05$, compared with the corresponding control group. ##, $p < 0.01$, compared with the corresponding control group. *, $p < 0.05$, compared with the corresponding CoCl₂- or insulin-treated groups. **, $p < 0.01$, compared with the corresponding CoCl₂- or insulin-treated groups. $n = 3$ for each group.

Fig. 3. Post-treatment of L-4F decreases HIF-1 α protein level and activity in CoCl₂- and insulin-treated OV208 cells. Cells were treated with CoCl₂ (100 μ M) or insulin (200 nM) for 24 h and then treated with vehicle or L-4F (10 μ g/ml) for an additional 1, 2, or 4 h. **A**, post-treatment of L-4F at 10 μ g/ml decreases HIF-1 α protein level in CoCl₂- and insulin-treated OV208 cells. **B**, post-treatment of L-4F at 10 μ g/ml for 4 h decreases CoCl₂- and insulin-induced increases of nuclear levels of HIF-1 α in OV208 cells. Cells were immunostained with a mouse monoclonal anti-HIF-1 α primary antibody and a goat anti-mouse IgG labeled with Alexa Fluor 568 (red fluorescence) as the secondary antibody. DAPI was used to stain nuclei (blue). Images are shown at the original magnification of 400 $\times$. Representative photographs of two independent experiments with similar results are shown. **C** and **D**, inhibition of HRE reporter gene transcription in CoCl₂- and insulin-treated cells by post-treatment of L-4F. OV208 cells were transfected with pGL3-Epo-HRE-Luc plasmid and grown in complete growth media for 24 h. After starvation overnight, cells were treated with CoCl₂ (100 μ M) or insulin (200 nM) for 24 h and then treated with L-4F (10 μ g/ml) for an additional 4 h (**C**) or 24 h (**D**). Luciferase activity was determined as described under *Materials and Methods*. **, $p < 0.01$, compared with the corresponding CoCl₂- or insulin-treated groups. $n = 3$ for each group.

of the transcription of downstream HIF-1 α target genes (Fig. 3C; Supplemental Fig. 2C).

L-4F Does Not Affect HIF-1 α Transcription. To determine whether L-4F affects HIF-1 α synthesis at the transcriptional level, we quantified HIF-1 α mRNA content to determine whether a change in HIF-1 α mRNA level precedes that of protein. Real-time RT-PCR analyses indicated that L-4F had no effect on the basal level of HIF-1 α mRNA (Supplemental Fig. 3A). Moreover, consistent with previous reports (Semenza, 2003; Pouyssegur et al., 2006; Lee et al., 2009), low oxygen and insulin did not affect HIF-1 α gene transcription (Supplemental Fig. 3B), suggesting that the regulation of HIF-1 α protein expression by L-4F occurs at the post-transcriptional level.

L-4F inhibits S6 Kinase Phosphorylation in an ERK-Dependent Manner. Activation of S6 kinase is critical for insulin-induced de novo synthesis of HIF-1 α (Semenza, 2003). To determine the molecular mechanism of HIF-1 α inhibition by L-4F, we tested whether L-4F affects the insulin-stimulated protein synthesis of HIF-1 α . Our data showed that L-4F at 10 μ g/ml prevented phosphorylation of S6 kinase (Fig. 4A). S6 kinase phosphorylation is regulated by the activation of upstream signaling molecules ERK and Akt. As

shown in Fig. 4B, L-4F inhibited activation of ERK1/2, but had no effect on the phosphorylation of Akt, except at 0.5 h, suggesting that the inhibition of S6 kinase activation may most likely be a result of the suppression of ERK phosphorylation. It is noteworthy that we did not observe an effect of CoCl₂ on the phosphorylation of ERK, Akt, and S6 kinase in OV208 cells (Supplemental Fig. 4A). This result is not surprising because CoCl₂ treatment mimics hypoxia, which leads to decreases in HIF-1 α protein degradation (Pouyssegur and Mechta-Grigoriou, 2006).

L-4F Treatment Promotes Proteasome-Dependent Protein Degradation. We next examined whether L-4F changes the stability of the HIF-1 α protein. CHX, a compound that prevents new protein synthesis, was used to inhibit de novo HIF-1 α protein synthesis. Our data showed that OV208 cells treated with CHX in combination with insulin exhibited a gradual decrease in HIF-1 α as a function of time, and simultaneous L-4F treatment accelerated the degradation of HIF-1 α protein (Fig. 5A). We observed a similar effect of L-4F on CoCl₂-treated OV208 cells (Supplemental Fig. 4B). Furthermore, MG-132, a proteasome inhibitor, led to a reversal of the inhibitory effect of L-4F on insulin-mediated HIF-1 α expression (Fig. 5B). These results

Fig. 4. Effect of L-4F on the insulin-stimulated activation of downstream signaling molecules in OV2008 cells. After an overnight starvation, OV2008 cells were treated with L-4F (10 μ g/ml) for 1 h, and insulin was added at a final concentration of 200 nM. Cell lysates were collected at various time points and subjected to Western blot analysis. A, L-4F inhibits insulin-stimulated phosphorylation of p70s6 kinase and subsequent HIF-1 α expression in OV2008 cells. B, effect of L-4F on insulin-stimulated phosphorylation of ERK1/2 and Akt in OV2008 cells.

suggest that L-4F inhibits insulin- and CoCl_2 -induced HIF-1 α expression and activity in ovarian cancer cells, in part, by accelerating the degradation of HIF-1 α protein.

Inhibition of Insulin- and CoCl_2 -Induced ROS Production by L-4F Treatment. It is reported that insulin treatment (Zhou et al., 2007; Lee et al., 2009) and CoCl_2 treatment (Chandel et al., 2000; Griguer et al., 2006) significantly increase cellular ROS levels, which subsequently promotes the synthesis of HIF-1 α and inhibits its degradation. As shown by dichlorofluorescein oxidation assay (Fig. 6), treatment of insulin and CoCl_2 led to an increase of cellular ROS levels in OV2008 cells. Pretreatment of L-4F dramatically prevented the cellular ROS production induced by insulin and CoCl_2 (Fig. 6), suggesting that the inhibitory role of L-4F on HIF-1 α expression may be a result of the inhibition of ROS accumulation.

Discussion

HIF-1 is a key cellular survival protein under hypoxia and is associated with tumor progression and metastasis in various solid tumors (Seeber et al., 2011). Targeting HIF-1 α could be an attractive anticancer therapeutic strategy (Se-

Fig. 5. Effect of L-4F on HIF-1 α protein stability in OV2008 cells. A, left, pretreatment of L-4F promotes HIF-1 α degradation in OV2008 cells. After an overnight starvation, OV2008 cells were treated with insulin (200 nM) for 3 h, L-4F (10 μ g/ml) for 1 h, and CHX (20 μ g/ml) for various durations. Cell lysates were collected and subjected to Western blot analysis. Representative data from three independent experiments with similar results are shown. Right, L-4F treatment promotes HIF-1 α degradation in OV2008 cells. After an overnight starvation, OV2008 cells were treated with insulin (200 nM) for 3 h and then treated with L-4F (10 μ g/ml) and CHX (20 μ g/ml) at the same time. Cell lysates were collected at various time points and subjected to Western blot analysis. Representative data from three independent experiments with similar results are shown. B, effect of pretreatment of L-4F on proteasome-mediated degradation of HIF-1 α in insulin-treated OV2008 cells. After an overnight starvation, OV2008 cells were treated with MG-132 (10 μ M) for 3 h, L-4F (10 μ g/ml) for 1 h, and insulin (200 nM) for an additional 4 h. Cell lysates were collected and subjected to Western blot analysis. Representative data from three independent experiments with similar results are shown.

Fig. 6. Effect of L-4F on CoCl_2 - and insulin-stimulated ROS production. OV2008 cells were pretreated with L-4F (10 $\mu\text{g}/\text{ml}$) for 1 h, and then treated with insulin (200 nM)/ CoCl_2 (100 μM) and DCFH-DA (10 μM) for 30 min. After washing cells twice with PBS, images of cells were captured with a fluorescence microscope. Representative figures are shown at the original magnification of 200 $\times$. A, L-4F inhibits insulin-stimulated ROS production in OV2008 cells. B, L-4F inhibits CoCl_2 -stimulated ROS production in OV2008 cells.

menza, 2003; Belozarov and Van Meir, 2005; Seeber et al., 2011). Expression of HIF-1 α is increased by both hypoxic and nonhypoxic stimuli. Low oxygen concentration or treatment with CoCl_2 , a hypoxic mimetic compound, inhibits the degradation of HIF-1 α and increases HIF-1 α protein stability and accumulation. Some growth factors, including insulin and LPA, also promote post-transcriptional protein synthesis and up-regulate the expression and activity of HIF-1 α (Semenza, 2003; Pouyssegur and Mechta-Grigoriou, 2006; Pouyssegur et al., 2006). In this article, we demonstrate that: 1) L-4F inhibits HIF-1 α expression in mouse tumor tissues (Fig. 1A); 2) pretreatment and post-treatment of L-4F and L-5F decrease low oxygen-, CoCl_2 -, insulin-, and LPA-induced expression and nuclear levels of HIF-1 α in human ovarian cancer cell lines (Figs. 1 and 3; Supplemental Figs. 1 and 2); and 3) L-4F inhibits CoCl_2 - and insulin-stimulated expression of HRE-driven reporter gene and activation of HIF-1 α target genes (Figs. 2 and 3; Supplemental Fig. 2). Real-time RT-PCR analyses indicated that L-4F has no effect on HIF-1 α gene transcription in OV2008 cells (Supplemental Fig. 3), indicating that the regulation of HIF-1 α protein by L-4F occurs at the post-transcriptional level.

There is compelling evidence that ROS are key players in the regulation of HIF-1 α under normoxia as well as hypoxia (Pouyssegur and Mechta-Grigoriou, 2006). As reported previously, treatment of cells with low oxygen concentration (Chandel et al., 2000; Guzy et al., 2005; Guzy and Schumacker, 2006), CoCl_2 (Chandel et al., 2000; Griguer et al., 2006), insulin (Zhou et al., 2007; Lee et al., 2009), and LPA (Chen et al., 1995; Saunders et al., 2010) lead to ROS gener-

ation. ROS production is critical for HIF-1 α expression in cells, and removal of ROS impairs HIF-1 α accumulation induced by hypoxia and insulin (Brunelle et al., 2005; Mansfield et al., 2005; Carnesecchi et al., 2006; Biswas et al., 2007). Ganapathy et al. (2012) reported that D-4F, an apoA-I mimetic peptide, significantly decreases the production of superoxide and H_2O_2 and improves the oxidative status on ID8 cells. However, it is unknown whether peptide treatment affects hypoxia- or growth factor-mediated ROS production. Here, we report that L-4F treatment dramatically inhibits insulin- and CoCl_2 -induced ROS production in OV2008 cells (Fig. 6). Furthermore, L-4F accelerated HIF-1 α degradation in cancer cells exposed to insulin and CoCl_2 (Fig. 5A; Supplemental Fig. 4B). MG-132, a 26S proteasome inhibitor, reversed the inhibitory effect of L-4F on insulin-mediated HIF-1 α expression (Fig. 5B). Taken together, these data demonstrate that L-4F decreases the protein stability of HIF-1 α and inhibits the accumulation of transcriptionally active HIF-1 α , at least in part, through its ROS-scavenging effect.

In an effort to find the molecular mechanism of HIF-1 α inhibition, we determined whether L-4F affects the synthesis of HIF-1 α protein. Insulin activates receptor tyrosine kinase and downstream signaling molecules, most notably S6 kinase, leading to increases of mRNA translation and de novo synthesis of HIF-1 α (Treins et al., 2002; Semenza, 2003). L-4F inhibits insulin-stimulated phosphorylation of S6 kinase at various time points, resulting in a decrease of HIF-1 α protein level (Fig. 4A). Further experiments showed that down-regulation of S6 kinase activity may be a result of the inhibition of the activation of ERK1/2, but not Akt (Fig. 4B). It is noteworthy that it is reported that ROS is involved in insulin-stimulated phosphorylation of ERK1/2 and S6 kinase, but not Akt (Zhou et al., 2007), indicating that ROS removal may also be involved in the inhibition of the de novo synthesis of HIF-1 α by L-4F.

Previous reports showed that D-4F (an apoA-I mimetic peptide identical to L-4F but synthesized with all D amino acids) increases the expression and activity of two antioxidant enzymes, heme oxygenase 1 and extracellular superoxide dismutase (SOD), in aorta from control and diabetic rats (Kruger et al., 2005). More recently, we also demonstrated that D-4F up-regulates the antioxidant enzyme Mn-SOD in ID8 cells, and knockdown of Mn-SOD results in the complete loss of antitumor effects of D-4F in a mouse ovarian cancer model (Ganapathy et al., 2012). Because SOD activity modulates ROS production and cellular oxidative stress, induction of SOD may be an important part of the mechanism of action of apoA-I mimetic peptides.

In conclusion, our data demonstrate that apoA-I mimetic peptides inhibit the expression and activity of HIF-1 α both in vivo and in cell culture. The inhibition of HIF-1 α may be a critical mechanism responsible for the suppression of tumor progression by apoA-I mimetic peptides.

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Authorship Contributions

Participated in research design: Gao, Reddy, and Farias-Eisner.
Conducted experiments: Gao, Chattopadhyay, Grijalva, and Su.

Contributed new reagents or analytic tools: Navab, Fogelman, Reddy, and Farias-Eisner.

Performed data analysis: Gao.

Wrote or contributed to the writing of the manuscript: Gao, Reddy, and Farias-Eisner.

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