Oltipraz and dithiolethione congeners inhibit hypoxia-inducible factor-1α activity through p70 ribosomal S6 kinase-1 inhibition and H2O2-scavenging effect

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Abstract

Hypoxia-inducible factor-1α (HIF-1α) induces tumor proliferation, angiogenesis and metastasis. Reactive oxygen species, hypoxia, and growth factor stimulation induce HIF-1α, and the augmented HIF-1α activity confers upon cancer cells the ability to adapt to microenvironments. Oltipraz is a cancer chemopreventive agent and has an inhibitory effect on angiogenesis and tumor growth. Nonetheless, the molecular mechanism of tumor inhibition is as yet unclear. This study investigated whether oltipraz and its congeners inhibit HIF-1α activity and, if so, the molecular basis of inhibition. Oltipraz and other 1,2-dithiole-3-thiones have the ability to prevent insulin- or hypoxia-induced HIF-1α expression through an increase in ubiquitination, thereby accelerating HIF-1α degradation and inhibiting HIF-1α–dependent gene transcription. Transfection of cells with a constitutively active mutant of p70 ribosomal S6 kinase-1 (CA-S6K1) increased the basal and insulin-inducible HIF-1α activity. CA-S6K1 overexpression reversed HIF-1α inhibition by rapamycin (a mammalian target of rapamycin/S6K1 inhibitor). However, the inhibitory effect of oltipraz on HIF-1α was not reversed by CA-S6K1 despite its S6K1 inhibition. The failure of dominant negative mutant AMP-activated protein kinase-α to restore the ability of insulin to increase HIF-1α against oltipraz excluded the possible role of AMP-activated protein kinase activation in the action of oltipraz. Oltipraz treatment abrogated insulin-induced H2O2 production, thereby preventing H2O2-enhanced HIF-1α expression and promoting its ubiquitination and degradation. In an animal model, tumor regression by oltipraz was accompanied by decreases in microvessel density and vascular endothelial growth factor induction. Oltipraz inhibits HIF-1α activity and HIF-1α–dependent tumor growth, which may result from a decrease in HIF-1α stability through S6K1 inhibition in combination with an H2O2-scavenging effect. [Mol Cancer Ther 2009;8(10):2791–802]

Introduction

Hypoxia-inducible factor-1α (HIF-1α) is a heterodimeric complex composed of the inducible subunit HIF-1α and the constitutively expressed HIF-1β (also known as “aryl hydrocarbon nuclear translocator”; ref. 1). HIF-1α is regulated by either an oxygen-dependent or -independent mechanism. Insulin and other ligands that activate receptor tyrosine kinases (e.g., insulin receptor α and insulin-like growth factor receptor α) increase the accumulation of HIF-1α under normoxic conditions. Activation of HIF-1α contributes to cancer progression through initiation of gene transcription, the protein products of which are required for angiogenesis, glucose transport, and cell survival (2–4). Hence, activated HIF-1α regulates genes involved in the adaption of cancer cells to unfavorable tumor microenvironments where the supply of oxygen and nutrients are limited (3, 5, 6). In fact, accumulation of HIF-1α is observed in a myriad of human cancers. Moreover, cancer cells that have augmented HIF-1α levels become aggressive and acquire resistance against chemotherapeutic agents (5, 7).

An increasing number of studies has described a series of compounds, such as YC-1, rapamycin, PX-478, and 17-AAG, that inhibit HIF-1α activity in cellular and animal models (8–11). Because some of these agents may exhibit toxicity or other adverse effects (9, 11), current pharmacologic interventions in HIF-1α activity are limited and expected to be advanced by tailor-made drugs. A class of 1,2-dithiole-3-thione compounds, of which oltipraz represents a prototype, has been extensively studied as cancer chemopreventive agents in many experimental models (12). Oltipraz induces diverse phase 2 enzymes, including glutathione S-transferase, UDP-glucurononyltransferase, and heme oxygenase-1, which contribute to decreasing the formation of carcinogen-DNA adducts. In clinical trials conducted in Qidong, China, oltipraz exerted beneficial effects in phase 1 and 2 metabolism of aflatoxin B1 (13). Moreover, oltipraz possesses...
diverse pharmacologic effects, such as reduced tumorigenesis, decreased liver fibrosis, and enhanced cell survival (12, 14, 15), suggesting that oltipraz has varying sensitivities to the growth of cells depending on the growth factor milieu.

The binding of insulin to insulin receptor in the plasma membrane disseminates signals that lead to physiologic responses. Insulin affects the stability of the HIF-1α protein by generating reactive oxygen species, in particular H₂O₂ (16, 17). H₂O₂ inactivates proline hydroxylase–domain enzymes, which initiate ubiquitination and degradation, thereby stabilizing HIF-1α (17). It has also been shown that insulin promotes HIF1A mRNA translation and thus increases de novo synthesis of HIF-1α through the mammalian target of rapamycin/p70 ribosomal S6 kinase-1 (S6K1) pathway (18). Because S6K1 enhances translation of HIF1A mRNA through 5′ terminal oligopyrimidine sequences in the HIF1A mRNA, insulin-dependent induction of HIF-1α is suppressed by treatment with rapamycin, a mammalian target of rapamycin–S6K1 inhibitor (19). Treatment with oltipraz or 1,2-dithiole-3-thione congeners activates AMP-activated protein kinase, which prevents insulin resistance through S6K1 inhibition (20). Oltipraz inhibition of S6K1 prevents the development of insulin resistance and hyperglycemia (21). Nevertheless, it is as yet unclear whether S6K1 inhibition and/or AMP-activated protein kinase activation by oltipraz contribute(s) to HIF-1α suppression.

In view of the roles of S6K1 and/or AMP-activated protein kinase in the regulation of HIF-1α activity and the known action of oltipraz on the AMP-activated protein kinase–S6K1 pathway, this study explored the effects of oltipraz and 1,2-dithiole-3-thione congeners on HIF-1α activity and HIF-1α-dependent gene induction in cellular and tumor-xenograft models. We examined whether these agents inhibit increased HIF-1α activity following insulin treatment and the underlying molecular basis of the inhibition. Here, we found that oltipraz accelerates degradation of ubiquitinated HIF-1α. Because the ability of oltipraz to repress HIF-1α activity was not reversed by either a constitutively active mutant of S6K1 or a dominant negative mutant of AMP-activated protein kinase, we investigated other mechanisms to explain the oltipraz-induced inhibition of HIF-1α activity. Our findings indicate that H₂O₂ production and H₂O₂-mediated HIF-1α stabilization by insulin were both inhibited by oltipraz, which enabled us to identify 1,2-dithiole-3-thiones as a novel class of compounds that inhibit HIF-1α through S6K1 inhibition in conjunction with an H₂O₂-scavenging activity. Finally, the in vivo results of the xenograft-tumor model confirmed the efficacy of oltipraz in the repression of tumor growth and inhibition of angiogenesis.

### Materials and Methods

#### Cells and Cell Culture Conditions

HT29 and HCT116, human colon cancer cell lines, were purchased from American Type Culture Collection. The cells were maintained in growth medium containing DMEM, 10% fetal bovine serum, and 1% penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. For all experiments, cells were grown to 80% to 90% confluence and were deprived of serum for 16 h before treatment. To create hypoxic conditions, cells were transferred to a hypoxic chamber (Forma Scientific), where they were maintained at 37°C in an atmosphere containing 5% CO₂, 1% O₂, and 94% N₂.

#### Chemicals and Reagents

Oltipraz (CJ12064); 5-(6-methoxypyrzinyl)-4-methyl-1,2-dithiole-3-thione (CJ11842); 5-benzo[b]thiophene-3-yl-1,2-dithiole-3-thione (CJ11788); 4,5,6,7-tetrahydrobenzo-1,2-dithiole-3-thione (CJ11784); 5-benzoi[b]biophene-3-yl-1,2-dithiole-3-thione (CJ11840); 4-methyl-5-phenyl-1,2-dithiole-3-thione (CJ11842); 5-(6-methoxyppyrazinyl)-4-methyl-1,2-dithiole-3-thione (CJ12064); and 5-(6-methoxyppyrazinyl)-4-ethyl-1,2-dithiole-3-thione (CJ12073) were synthesized as described previously (20).

#### Immunoblot Analysis

Cell lysates were prepared according to previously published methods (22). Briefly, cells were lysed in buffer containing 10 mmol/L Tris–HCl (pH 7.1), 100 mmol/L NaCl, 1 mmol/L EDTA, 10% glycerol, 0.5% Triton X-100, 0.5% Nonidet P-40, 1 mmol/L dithiolethione, and 0.5 mmol/L phenylmethylsulfonyl fluoride, which was supplemented with a protease inhibitor cocktail (Calbiochem). SDS-PAGE and immunoblot analyses were done as described previously (20). Immunoreactive protein was visualized by the ECL chemiluminescence detection kit (Amersham Biosciences). Equal loading of proteins was verified by immunoblotting for β-actin. Scanning densitometry of the immunoblots was done with the Image Scan and Analysis System (Alpha Innotech Corp.). The total area of each lane was integrated, and band intensity was assessed using AlphaEase version 5.5 software, followed by background subtraction.

#### Hypoxia Response Element Reporter Gene Assay

Hypoxia response element–A549 cell line was established by transfection of hypoxia response element–luciferase reporter plasmid (23) into the human lung carcinoma cell line, A549, using Lipofectamin Plus (Invitrogen) and subsequent selection by treatment with G418 (600 μg/mL; GIBCO). Hypoxia response element–A549 cells were incubated in DMEM. Following overnight serum deprivation, the cells were exposed to 100 mmol/L insulin for 24 h at 37°C with or without 30 μmol/L oltipraz or each 1,2-dithiole-3-thione congener. Luciferase activity was measured by adding luciferase assay reagent (Promega).
Figure 1. Inhibition of insulin, hypoxia, or CoCl2 induction of HIF-1α by oltipraz and 1,2-dithiole-3-thione congeners. A, inhibition of insulin, hypoxia, or CoCl2 induction of HIF-1α by oltipraz. HT29 cells were incubated in a medium containing 100 nmol/L insulin for the indicated periods after treatment with vehicle or oltipraz for 1 h (top). Oltipraz treatment inhibited the induction of HIF-1α (100 nmol/L insulin; 3 h) in a concentration-dependent manner (middle). The cells were also incubated under the condition of hypoxia (1% oxygen) or in a medium containing 100 μmol/L CoCl2 for the indicated periods following vehicle or oltipraz treatment for 1 h (bottom). B, the chemical structures of novel synthetic 1,2-dithiole-3-thione congeners. C, inhibition of insulin (left) or hypoxia (right) induction of HIF-1α by novel 1,2-dithiole-3-thiones. The expression of HIF-1α was determined in lysates of HT29 cells that had been exposed to insulin or hypoxia for 6 h following treatment with each 1,2-dithiole-3-thione (30 μmol/L; 1 h). The relative band intensity of HIF-1α to HIF-1β was assessed by scanning densitometry of the immunoblots. Values are mean ± SE from five independent experiments [treatment mean significantly different from vehicle-treated control (**, P < 0.01) or insulin or hypoxia (#, P < 0.05; ##, P < 0.01)]. NS, not significant; WCL, whole cell lysates. D, the concentration-dependent inhibition of insulin induction of HIF-1α by 1,2-dithiole-3-thione congeners. Values are mean ± SE from five independent experiments.
Real-time RT-PCR Assay

Total RNA was isolated from cells using Trizol (Invitrogen), and cDNA was synthesized by reverse transcription using an oligo(dT) primer. Then, real-time PCR was done with a Light Cycler 1.5 (Roche) using a Light Cycler DNA master SYBR green-1 kit, according to the manufacturer’s instructions. PCR was done using primers selective for the genes encoding vascular endothelial growth factor (VEGF; sense, 5′-CCAAGAGTTTGCTCTTCAAC-3′; antisense, 5′-AGAGGCGTGGCATACAGGTT-3′; ref. 24), glucose transporter 1 (sense, 5′-CGGGCCAGAGTGTGCTAAA-3′; antisense, 5′-TGACGATCCGGAGCCAAATG-3′; ref. 25), aldolase-A (sense, 5′-TGCTACTACCAGCACCACG-3′; antisense, 5′-ATGCTCCCAGTGACCTAC-3′; ref. 26), HIF-1α (sense, 5′-GTCGCCAGACCGTCAACAGGCA-3′; antisense, 5′-GTAAAAGTGGACACGAGC-3′; ref. 27), and β-actin (sense, 5′-CTCTCCAGCCTTCTTCG-3′; antisense, 5′-CAGCACTGTGGTTGGCAGTACG-3′; ref. 28).

Measurement of Cellular H2O2

Dichlorofluorescein diacetate is a cell-permeable nonfluorescent probe that is cleaved by intracellular esterases and oxidized primarily by H2O2, producing the fluorescent dichlorofluorescein. The level of H2O2 generation was determined by the concomitant increase in dichlorofluorescein fluorescence. HT29 cells were incubated with 100 nmol/L oltipraz treatment for 30 min. The cells were then stained with 10 μmol/L dichlorofluorescein diacetate for 30 min at 37°C, and the fluorescence intensity in the cells was measured using Leica TCS NT confocal microscope (Leica Microsystems).

Transient Transfection

The cells (5 × 10⁵ cells/well) were plated in six-well plates overnight, serum-starved for 3 h, and transfected with a plasmid encoding a constitutively active form of S6K1 (CA-S6K1) or His-tagged ubiquitin for 6 h in the presence of FuGENE HD Reagent (Roche). Transfected cells were then incubated in Eagle’s minimum essential medium containing 1% fetal bovine serum for 18 h.

Recombinant Adenoviral Dominant Negative Mutant of AMP-Activated Protein Kinase-α

The plasmid encoding a dominant negative mutant of AMP-activated protein kinase-α was kindly provided by Dr. J. Ha (Kyung Hee University, Korea). HCT116 cells were infected with the adenovirus diluted in DMEM containing 10% fetal bovine serum at a multiplicity of infection of 50 and incubated for 24 h. After removal of the viral suspension, the cells were further maintained with DMEM containing 10% fetal bovine serum for 1 d, followed by treatment with reagents as indicated in the figure legends. Adenoviral green fluorescent protein (Newgex) was used as an infection control. The efficiency of infection was consistently >90% using this method.

Immunoprecipitation Assay

To assess HIF-1α ubiquitination, cells were transfected with the plasmid of His-tagged ubiquitin. Cell lysates were incubated with anti-His antibody overnight at 4°C. After immunoprecipitation, samples were resolved and were subjected to SDS-PAGE. The samples were immunoblotted with anti-ubiquitin antibody. The antigen-antibody complex was precipitated following incubation for 2 h at 4°C with protein G–agarose. The immune complex was solubilized in 2× Laemmli buffer and boiled for 5 min. Samples were resolved and analyzed using 7.5% SDS-PAGE and then transferred to nitrocellulose membrane. The samples were then immunoblotted with the antibody directed against His or HIF-1α.

Tumor-Xenograft Animal Model

Animal studies were conducted in accordance with the institutional guidelines for care and use of laboratory animals at Seoul National University. HCT116 cells were cultured at the density of 2 × 10⁶ per dish, harvested, and injected s.c. into BALB/c nude mice in the right flank. Mice with a tumor volume >100 mm³ were selected 10 d after the injection and randomly divided into two groups (n = 7 in each group). Oltipraz (200 mg/kg body weight) dissolved in 40% polyethylene glycol 400 was orally administered to mice thrice per week for 2 wk, followed by three consecutive daily treatments. Tumor volume was measured using a caliper.

Immunohistochemistry

Tumor tissues were fixed in 10% formalin, embedded in paraffin, cut into 4-μm-thick sections, and mounted on slides. Tissue sections were immunostained with the antibody directed against CD31 or VEGF. Microvessel density in tumor tissue was quantified according to a previously published method (29).

Data Analysis

One-way ANOVA procedures were used to assess significant differences among treatment groups. For each significant treatment effect, the Newman-Keuls test was used to compare multiple group means.

Results

Inhibition of Insulin Induction of HIF-1α by Oltipraz or 1,2-Dithiole-3-thione Congeners

Insulin induces HIF-1α accumulation through protein stabilization as well as de novo protein synthesis (5, 16, 17). First, we examined whether oltipraz treatment inhibits the induction of HIF-1α by insulin (100 nmol/L for 3 hours) in HT29 cells, a human colon cancer cell line. Oltipraz had an inhibitory effect on HIF-1α protein synthesis (5, 16, 17). Then, we incubated the cells with insulin and examined the HIF-1α protein level. The results show that oltipraz inhibited the induction of HIF-1α by insulin (Fig. 1A, top and middle; oltipraz is nontoxic at the concentrations). Oltipraz treatment also inhibited HIF-1α activation stimulated by either hypoxia or CoCl2 (Fig. 1A, bottom). HIF-1α is expressed as a full-length (826 amino acids) or smaller form (735 amino acids; ref. 30). The small one is generated from its mRNA by alternative splicing and is less active because it lacks C-terminal transactivation domain. The arrow head indicates the full-length form.

Next, we comparatively evaluated the effects of synthetic 1,2-dithiole-3-thione congeners on HIF-1α induction by insulin or hypoxia (Fig. 1B and C). Treatment of HT29 cells

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with each 1,2-dithiole-3-thione derivative (30 μmol/L for 1 hour) variably inhibited the induction of HIF-1α by insulin (Fig. 1C, left) or hypoxia (Fig. 1C, right); CJ11764, CJ11766, and CJ12073 had strong inhibitory effects, whereas CJ11788, CJ11792, and CJ12064 had moderate effects. The effects of CJ11780, CJ11840, and CJ11842 were minor. Among these compounds, six 1,2-dithiole-3-thione congeners (CJ11764, CJ11766, CJ11788, CJ11792, CJ12064, and CJ12073) were chosen for subsequent experiments; these compounds exhibited inhibitory effects on insulin-induced HIF-1α activation in a concentration-dependent manner (Fig. 1D).

**Inhibition of HIF-1α-Dependent Gene Transcription by Oltipraz**

HIF-1α forms a heterodimer with its binding partner HIF-1β, which then moves into the nucleus and mediates gene induction (2). The levels of nuclear HIF-1α in HT29 cells treated with insulin or insulin + oltipraz were measured. Oltipraz almost completely prevented the ability of insulin to increase nuclear HIF-1α content for the periods examined (Fig. 2A). Consistently, oltipraz treatment abrogated insulin-induced increases in mRNA levels of HIF-1α target genes, specifically VEGF, glucose transporter 1, and aldolase-A (Fig. 2B). The effects of insulin were consistent with the previous observations (28, 31). To determine whether oltipraz inhibits HIF-1α-driven gene transactivation, reporter gene assays were conducted in A549 cells that had been stably transfected with a hypoxia response element reporter gene construct. Hypoxia response element–A549 cells that had been stably transfected with the hypoxia response element–luciferase construct were incubated with insulin for 24 h following oltipraz treatment for 1 h. Luciferase activity was measured in the cell lysates. D, inhibition of hypoxia response element reporter gene induction by 1,2-dithiole-3-thione congeners. Luciferase activity was measured as described in C. Values are mean ± SE from five independent experiments (treatment mean significantly different from vehicle-treated control (**, P < 0.01) or insulin (#, P < 0.05; ##, P < 0.01)).
Real-time RT-PCR analyses indicated that oltipraz had no effect on the level of HIF-1α mRNA, whereas actinomycin D (ActD), an inhibitor of gene transcription, was used as a positive control. A, the effect of oltipraz on HIF-1α protein stability. HT29 cells that had been incubated with insulin or insulin + oltipraz (Olt) were treated with cycloheximide (CHX; 20 μg/mL) for the indicated periods and were subjected to immunoblots for HIF-1α or HIF-1β. Values are mean ± SE from five independent experiments (treatment means significantly different from vehicle-treated control (**, P < 0.01) or insulin (#, P < 0.05; ##, P < 0.01)). B, proteasomal degradation of ubiquitinated HIF-1α in cells treated with insulin. HCT116 cells that had been transfected with the plasmid encoding His-tagged ubiquitin (His-Ubi) were treated with vehicle or MG132 (10 μmol/L) for 3 h and were subsequently treated with insulin (3 h) or insulin + oltipraz (4 h). HIF-1α immunoprecipitates were immunoblotted with anti-ubiquitin antibody. HIF-1β was immunoblotted in cell lysates. Values are mean ± SE from five independent experiments (significantly different from respective treatment with insulin + CHX). **, P < 0.01.

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Figure 3. Increase in proteasomal degradation of HIF-1α by oltipraz. A, the effect of oltipraz on HIF-1α mRNA level. HT29 cells were incubated with insulin or insulin + oltipraz (Olt) for 6 h, and HIF-1α mRNA levels in the cells were measured using real-time RT-PCR assays. Actinomycin D (ActD), an inhibitor of gene transcription, was used as a positive control. B, the effect of oltipraz on HIF-1α protein stability. HT29 cells that had been incubated with insulin or insulin + oltipraz were treated with cycloheximide (CHX; 20 μg/mL) for the indicated periods and were subjected to immunoblots for HIF-1α or HIF-1β. Values are mean ± SE from five independent experiments (treatment means significantly different from vehicle-treated control (**, P < 0.01) or insulin (#, P < 0.05; ##, P < 0.01)). C, proteasomal degradation of ubiquitinated HIF-1α in cells treated with insulin. HCT116 cells that had been transfected with the plasmid encoding His-tagged ubiquitin (His-Ubi) were treated with vehicle or MG132 (10 μmol/L) for 3 h and were subsequently treated with insulin (3 h) or insulin + oltipraz (4 h). HIF-1α immunoprecipitates were immunoblotted with anti-ubiquitin antibody. HIF-1β was immunoblotted in cell lysates. Values are mean ± SE from five independent experiments (significantly different from respective treatment with insulin + CHX). **, P < 0.01.
Figure 4. The role of S6K1 or AMP-activated protein kinase in the inhibition of HIF-1α by oltipraz. **A**, the effect of oltipraz on the insulin and hypoxia signaling pathway. HT29 cells were treated with insulin or insulin + oltipraz and were subjected to immunoblotting (top). HT29 cells were incubated in an atmosphere containing 1% O2 with or without oltipraz and were subjected to immunoblotting (bottom). Results were confirmed by four repeated experiments.

**B**, the effect of CA-S6K1 overexpression on HIF-1α levels (top). HT29 cells that had been transfected with a plasmid encoding for CA-S6K1 were treated with either oltipraz or rapamycin (Rapa) in conjunction with insulin for 3 h. Immunoblots for c-myc verified the transfection efficiency of CA-S6K1. The dose-response inhibitory effect of rapamycin on HIF-1α induction was also assessed in HT29 cells (bottom).

**C**, the effect of dominant negative mutant of AMP-activated protein kinase-α overexpression on HIF-1α levels. HCT116 cells that had been infected with adenoviral green fluorescent protein or adenoviral dominant negative mutant of AMP-activated protein kinase for 24 h were treated with insulin or insulin + oltipraz, as described in the legend to Fig. 1. Immunoblots for green fluorescent protein or HA confirmed overexpression of green fluorescent protein or dominant negative mutant of AMP-activated protein kinase-α. Values are mean ± SE from five independent experiments [treatment mean significantly different from vehicle-treated control (**, P < 0.01) or control treated with insulin (#, P < 0.05; ##, P < 0.01)].
oltipraz treatment, which matches the decrease in HIF-1α content by oltipraz (Fig. 3C, bottom), suggesting that oltipraz may facilitate HIF-1α ubiquitination. These results lend support to the contention that oltipraz inhibits insulin-inducible HIF-1α activity in cancer cells, which may result from increased degradation of HIF-1α protein.

The Role of S6K1 or AMP-Activated Protein Kinase in the Inhibition of HIF-1α by Oltipraz

Consistent with the previous report (20), oltipraz treatment inhibited insulin-induced phosphorylations of mammalian target of rapamycin or S6K1 but had no effect on Akt phosphorylation in HT29 cells (Fig. 4A, top). Moreover, oltipraz treatment under a hypoxic condition inhibited mammalian target of rapamycin and S6K1 phosphorylations to greater extents than did hypoxia alone (Fig. 4A, bottom). In view of the known effect of S6K1 on HIF-1α expression (5), we determined whether constitutive activation of S6K1 antagonizes the ability of oltipraz to decrease HIF-1α content. Apparently, transfection of the cells with a constitutive active mutant of S6K1 (CA-S6K1) significantly increased basal and insulin-inducible HIF-1α levels (Fig. 4B, top), showing that S6K1 regulates HIF-1α activity. However, the inhibitory effect of oltipraz on insulin-inducible HIF-1α was not reversed by CA-S6K1 transfection. Rapamycin,

Figure 5. Oltipraz inhibition of HIF-1α induction by H2O2. A, dichlorofluorescein oxidation. DCF fluorescence representing cellular H2O2 production was monitored in HT29 cells treated with insulin or insulin + oltipraz for 10 min. Reflection images of the same fields verify the shape of cells. Results were confirmed by three repeated experiments. B, the time-course effect of oltipraz on HIF-1α induction by H2O2. C, proteasomal degradation of ubiquitinated HIF-1α in cells treated with H2O2. HCT116 cells that had been transfected with the plasmid encoding His-tagged ubiquitin were treated with vehicle or MG132 (10 μmol/L; 3 h) and were subsequently treated with H2O2 (3 h) or H2O2 + oltipraz (4 h). HIF-1α immunoprecipitates were immunoblotted with anti-ubiquitin antibody. HIF-1β expression represents a loading control. Values are mean ± SE from five independent experiments [treatment means significantly different from vehicle-treated control (**, \( P < 0.01 \)) or H2O2 (##, \( P < 0.01 \)).

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which inhibits mammalian target of rapamycin–S6K1 activity by inducing the dissociation of mammalian target of rapamycin–raptor complex through binding FKBP12, also repressed the increase of HIF-1α level by insulin (32). The dose-response inhibitory effect of rapamycin on HIF-1α induction by insulin was confirmed as a control (Fig. 4B, bottom). This rapamycin inhibition of HIF-1α was reversed by CA-S6K1, indicating that S6K1 indeed activates

![Graph A: Tumor Volume and Body Weight](image)

![Graph B: p-mTOR and p-S6K1](image)

![Graph C: CD31 and VEGF](image)

![Graph D: Proposed Model](image)

**Figure 6.** Inhibition of tumor growth and angiogenic gene induction by oltipraz. **A,** inhibition of HCT116 tumor growth volume by oltipraz treatment. Nude mice that had been s.c. xenografted with HCT116 cells in the right flank were treated with vehicle or oltipraz (200 mg/kg/d p.o. for 14 d). Tumor volume (cubic millimeters) and body weight (grams) were monitored at 14 d. **B,** inhibition of mammalian target of rapamycin (S2448) and S6K1 (T421/T424) phosphorylations by oltipraz. HCT116 tumor tissues of mice were subjected to immunoblotting. The relative band intensity was assessed by scanning densitometry of the immunoblots. Immunoblotting for β-actin verified equal loading of proteins. **C,** inhibition of microvessel density and VEGF expression by oltipraz. Microvessel density was monitored by measuring immunohistochemical intensities of CD31 in HCT116 tumor tissues of mice that had been treated with vehicle or oltipraz for 14 d. The number of CD31 and VEGF-positive cells was counted in five randomly selected fields from each slide. Original magnification, ×400. **D,** a proposed model by which oltipraz inhibits inducible HIF-1α activity. Values are mean ± SE from seven animals (treatment mean significantly different from vehicle-treated control). *, P < 0.05; **, P < 0.01.
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HIF-1α. Hence, the inhibitory action of oltipraz on HIF-1α induction by insulin results at least in part from its inhibition of S6K1. Moreover, our results suggest that oltipraz may have an additional mechanism for inhibiting HIF-1α activity.

In HT29 cells, oltipraz treatment enhanced insulin-induced phosphorylations of AMP-activated protein kinase-α and acetyl-CoA carboxylase at 3 to 6 hours, and this was accompanied by commensurate decreases in S6 phosphorylation (Fig. 4A), confirming that oltipraz inhibits S6K1 by a mechanism involving AMP-activated protein kinase activation through TSC2 (20). In the subsequent experiment, we examined the role of AMP-activated protein kinase activation in the inhibitory action of oltipraz on HIF-1α in cells treated with insulin. The failure of a dominant negative mutant of AMP-activated protein kinase-α to restore the ability of insulin to induce HIF-1α against oltipraz excluded the possible role of AMP-activated protein kinase activation in the inhibitory action of oltipraz on HIF-1α (Fig. 4C). This contention was further supported by the finding that dominant negative mutant of AMP-activated protein kinase-α alone rather prevented the induction of HIF-1α by insulin (Fig. 4C). These results indicate that HIF-1α repression by oltipraz may result in part from S6K1 inhibition but not AMP-activated protein kinase activation, and this may not be the only mechanism of HIF-1α inhibition.

Inhibition of H2O2-Induced HIF-1α Stabilization by Oltipraz

Recently, it has been shown that insulin treatment increases H2O2, which promotes the stability of HIF-1α (33). In an effort to find an additional mechanism responsible for the inhibitory action of oltipraz on HIF-1α, the effects of oltipraz on the increased production of H2O2 by insulin and on the increased HIF-1α activation by H2O2 were determined in cells. Insulin exposure to HT29 cells increased the production of H2O2, as shown by the enhanced dichlorofluorescein fluorescent intensity (Fig. 5A). Oltipraz treatment completely prevented the ability of insulin to increase H2O2 production, as did polyethylene glycol–catalase treatment. Consistently, H2O2 treatment (0.5 mmol/L for 0.5 hours) greatly promoted the level of HIF-1α at least up to 3-hour time point. Simultaneous oltipraz treatment, however, almost completely abolished HIF-1α induction by H2O2 (Fig. 5B). The level of HIF-1β, which was monitored as a negative control, was unchanged. In HCT116 cells treated with H2O2, MG132 treatment caused accumulation of ubiquitinated HIF-1α, which was further increased by simultaneous oltipraz treatment (Fig. 5C). These data raised the possibility that oltipraz enhances ubiquitination of HIF-1α against H2O2, thereby increasing its degradation. Collectively, these results support the notion that oltipraz or novel 1,2-dithiole-3-thione congeners inhibit HIF-1α activity by facilitating ubiquitin-dependent degradation of HIF-1α against H2O2 produced in cells treated with insulin.

Inhibition of Tumor Growth and Angiogenesis

HIF-1α enhances new blood vessel formation, which represents a fundamental step in the creation of an independent blood supply for tumor development (4, 11). Hence, it is expected that oltipraz inhibition of HIF-1α antagonizes tumor growth and angiogenesis. Having identified HIF-1α inhibition by oltipraz, we examined the in vivo inhibitory effect of oltipraz on HIF-1α target gene expression and the consequent tumor growth inhibition in BALB/c nude mice transplanted with HCT116 cells. Once the tumor mass in xenografted animals was palpable (>150 mm³), the animals were treated with vehicle or oltipraz (n = 7 animals per group). Oltipraz treatment (200 mg/kg/d for 14 days) significantly diminished the tumor volume with no change in body weight (Fig. 6A). Moreover, oltipraz inhibited the phosphorylations of mammalian target of rapamycin and S6K1 in the tumor tissue (Fig. 6B). Microvessel density, which represents angiogenesis in tumor tissue, was also reduced by oltipraz treatment, as evidenced by a decrease in CD31 staining intensity (an endothelial marker; Fig. 6C, left). Similarly, oltipraz treatment inhibited VEGF expression in the tumor tissue (Fig. 6C, right). These results support the conclusion that oltipraz has the ability to inhibit HIF-1α activity at least in part through scavenging H2O2 produced by insulin and that oltipraz inhibition of HIF-1α was sufficient to inhibit tumor growth and angiogenesis in a xenograft animal model (Fig. 6D).

Discussion

HIF-1α is a member of the bHLH (basic helix-loop-helix) and PAS (Per-ARNT-Sim) families. The bHLH and PAS domains are required for HIF-1α to form a complex with HIF-1β (34). This complex recognizes HIF–specific DNA sequences in the hypoxia response element of target genes (34). HIF-1α has two transactivation domains, N-terminal and C-terminal. The C-terminal domain especially binds with transcription coactivators such as CREB binding protein/p300 when HIF-1 interacts with hypoxia response element in the target gene promoter (3). Activation of HIF-1α contributes to cancer progression through initiating angiogenic gene transcription. Therefore, HIF-1α is considered as a promising target against cancer development and growth.

A series of anticancer agents have been shown to inhibit HIF-1α (9, 10). Moreover, additional new molecules are being studied for HIF-1α inhibition. Unfortunately, however, many of these anticancer agents or drug candidates do not inhibit HIF-1α selectively, and not infrequently, their HIF-1α inhibitory concentrations are higher than those that inhibit tumor growth (9, 11). Moreover, toxicities might be severe or as yet not tested. The chemopreventive capacity and toxicity of oltipraz have been studied in a variety of animal models (e.g., colon carcinogenesis; ref. 12). In addition, oltipraz has the ability to inhibit tumor growth and angiogenesis in an angiosarcoma xenograft model (35). However, how oltipraz inhibits angiogenesis at the molecular level had not been established. In this article, we have shown for the first time that oltipraz has an inhibitory effect on HIF-1α induction by insulin and verified its ability of tumor growth inhibition. Moreover, this study identified other 1,2-dithiole-3-thione congeners with HIF-1α-inhibition efficacy. Our results provide evidence that oltipraz prevents the ability of insulin to induce HIF-1α–driven gene transcription. Consistently, oltipraz decreased the levels of HIF-1α–inducible gene transcription. Published OnlineFirst September 29, 2009; DOI: 10.1158/1535-7163.MCT-09-0420
transcripts. In addition, 1,2-dithiole-3-thione congeners exhibited similar inhibitory actions on hypoxia response element activity, supporting the concept that oltipraz and its congeners repress HIF-1α-dependent gene transactivation by decreasing the nuclear level of HIF-1α. Overall, the 1,2-dithiole-3-thione moiety seemed to have the ability to inhibit HIF-1α. Hence, these compounds are likely to inhibit the adaptation of cancer cells to tumor microenvironments limited in oxygen and nutrients.

The oxygen-dependent degradation domain is essential for HIF-1α regulation (8). The oxygen-dependent degradation domain has proline residues hydroxylated by proline hydroxylase–domain protein, which recruits the E3 ubiquitin ligase complex (8). Thus, HIF-1α is continuously degraded by the ubiquitin–proteasome system under normoxic conditions. Insulin inactivates proline hydroxylase–domain protein through H2O2 production (36, 37) and the subsequent oxidation of ferrous iron. Moreover, oncogenes, growth factors, and cytokines induce HIF-1α by stabilizing the protein or augmenting de novo protein synthesis (5). In the present study, oltipraz promoted HIF-1α ubiquitination in cancer cells exposed to insulin. Our data showing a substantial increase in HIF-1α ubiquitination by oltipraz correlate well with a decrease in nuclear HIF-1α. MG132 treatment causes accumulation of ubiquitinated HIF-1α by inhibiting HIF-1α degradation through the 26S proteasome system. The hypothesis that oltipraz facilitates HIF-1α ubiquitination and degradation is strengthened by our data, showing that oltipraz accelerates HIF-1α degradation with no change in HIF-1α mRNA. Factor inhibiting hypoxia-inducible factor hydroxylates an aspartyl residue in C-terminal activation domain, whereas arrest defective 1 acetylates lysine (3). These modifications facilitate the interaction between HIF-1α and the E3 ubiquitin ligase complex, leading to disassembly of HIF-1α from the 26S proteasome. Hypoxic conditions prevent proline hydroxylase–domain protein from hydroxylating proline residues because of oxygen deficiency (36, 37). Our results showed that oltipraz and 1,2-dithiole-3-thione congeners also inhibit HIF-1α activation stimulated by either hypoxia or CoCl2 (Fig. 1A and C), suggesting that the target of oltipraz that leads to accelerated HIF-1α degradation may lie downstream of the ubiquitination step. The exact target for HIF-1α inhibition remains to be clarified.

Studies have shown that S6K1 is involved in the regulation of HIF-1α (9). Insulin promotes HIF-1A mRNA translation through S6K1 and thus increases de novo synthesis of HIF-1α protein and (b) S6K1 enhances translation of HIF1A mRNA through 5’ terminal oligopolyuridymined sequences (18, 19). In this study, CA-S6K1 transfection reversed the ability of rapamycin to repress insulin-induced HIF-1α activity, confirming that S6K1 indeed increases HIF-1α activity. Rapamycin inhibits S6K1 by dissociating mammalian target of rapamycin–raptor complex through binding FKBP12 (38). Oltipraz treatment unchanged the expression of FKBP12 and did not increase mammalian target of rapamycin–FKBP12 complex formation (20), suggesting that the target of oltipraz differs from that of rapamycin. Oltipraz and its congeners activate AMP-activated protein kinase, which causes S6K1 inhibition through TSC2 (20). As expected, oltipraz inhibited insulin-induced phosphorylations of mammalian target of rapamycin and S6K1 in HT29 cells. Hence, it is expected that S6K1 inhibition by oltipraz contributes to HIF-1α inhibition. Moreover, our in vivo study confirmed decreases in mammalian target of rapamycin and S6K1 phosphorylations in the xenograft tumor tissue, strengthening the concept that oltipraz represses HIF-1α in association with mammalian target of rapamycin and S6K1 inhibition. However, the inhibitory effect of oltipraz on insulin-induced HIF-1α activity was not antagonized by CA-S6K1, suggesting that an additional mechanism may still exist.

AMP-activated protein kinase is also implicated with HIF-1α activity (39, 40). AMP-activated protein kinase activation by either aminomimidazole carboxamide ribonucleotide or metformin antagonized HIF-1α induction by insulin or insulin-like growth factor, suggesting that chemical activation of AMP-activated protein kinase might be associated with HIF-1α inhibition (41). In cells treated with oltipraz, dominant negative mutant of AMP-activated protein kinase-α transfection failed to restore the ability of insulin to induce HIF-1α. Therefore, AMP-activated protein kinase activation by oltipraz is unlikely to directly contribute to HIF-1α inhibition. This possibility is further supported by the finding that dominant negative mutant of AMP-activated protein kinase-α transfection rather abolished HIF-1α induction by insulin. Because chemical activation of AMP-activated protein kinase leads to S6K1 inhibition, it is plausible that S6K1 inhibition elicited by AMP-activated protein kinase activation contributes to HIF-1α inhibition. Our results support the contention that the capability of oltipraz to repress HIF-1α may result in part from S6K1 inhibition, and this may not be the only mechanism for oltipraz-induced HIF-1α inhibition. This can be explained by studies from our laboratory and those from Biswas et al. (33), showing that insulin treatment increased H2O2 production, which promoted the stability of HIF-1α, and that oltipraz completely prevented cellular H2O2 production by insulin and thereby inhibited HIF-1α stabilization by H2O2. So, oltipraz prevents the ability of insulin to increase H2O2 and facilitates ubiquitin-dependent HIF-1α degradation. Our data corroborates the cancer chemopreventive effect of oltipraz, which may be associated at least in part with its increased phase 2 enzyme induction and antioxidant capacity (42–45).

In summary, oltipraz inhibits HIF-1α activity and HIF-1α–dependent gene transcription, which may result from a decrease in HIF-1α stability through S6K1 inhibition in conjunction with an H2O2-scavenging effect. Consistent with HIF-1α inhibition, oltipraz suppressed tumor growth in an animal model. In the tumor tissue, oltipraz decreased microvessel density and VEGF induction, which rely on HIF-1α induction. Pharmacokinetic studies showed that the plasma concentration of oltipraz (Cmax) was 3.5 μmol/L in mice after oral administration (50 mg/kg; ref. 46). The predicted plasma concentration of oltipraz is 14 μmol/L in our model (200 mg/kg). Thus, the IC50 (the half maximal inhibitory concentration) value (~10 μmol/L) of oltipraz for HIF-1α inhibition seems to be physiologically relevant.
Our findings showing the in vivo antitumor effect introduce the application of oltipraz and 1,2-dithiole-3-thione congeners as a new class of HIF-1α inhibitors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Oltipraz and dithiolethione congeners inhibit hypoxia-inducible factor-1 α activity through p70 ribosomal S6 kinase-1 inhibition and H$_2$O$_2$-scavenging effect

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