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6-(1-Oxobutyl)-5,8-dimethoxy-1,4-naphthoquinone inhibits Lewis lung cancer by antiangiogenesis and apoptosis

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Lung cancer is a leading cause of cancer mortality worldwide. Novel and nontoxic agents targeting angiogenesis and tumor cell proliferation and survival are desirable for lung cancer chemoprevention and treatment. Previously we have reported that 6-(1-oxobutyl)-5,8-dimethoxy-1,4-naphthoquinone (OXO) exhibits anti-tumor activity against S-180 sarcoma *in vitro* and *in vivo*. Here we studied the anti-angiogenic and apoptogenic attributes of OXO *in vitro* and *in vivo* targeting lung cancer. In human umbilical vein endothelial cells (HUVECs), we show that OXO more potently inhibited VEGF-stimulated than basic bFGF-stimulated HUVEC proliferation and capillary differentiation. In Lewis lung carcinoma (LLC) cells, OXO not only induces S-phase arrest and mitochondria/caspase-9 pathway mediated apoptosis, but also effectively down-regulated the hypoxia-induced expression of HIF-1α and VEGF at mRNA and protein levels in LLC and decreased VEGF secretion into conditioned culture media. OXO significantly reduced *in vivo* functional angiogenesis in the mouse Matrigel plug assay. Furthermore, OXO potently inhibited the growth of LLC cells inoculated on the flank of syngenic mice at dosages that did not affect their body weight. The *in vivo* anti-cancer effect was associated with decreased HIF-1α and VEGF expression, decreased microvessel density as well as a reduction of tumor cell proliferation and increased tumor cell apoptosis. Taken together, these results demonstrate that OXO exerts anti-cancer activity through anti-angiogenesis and tumor cell cycle arrest and apoptosis. These findings warrant additional studies of OXO as a novel agent for the chemoprevention and treatment of lung cancer.
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Key words: angiogenesis; HIF-1 alpha; VEGF; apoptosis; lung cancer

Lung cancer is the leading cause of cancer mortality worldwide,[1] including the United States (US).[2] The survival benefit of current cytotoxic cancer therapeutic drugs is very dismal in spite of serious drug side effects. As in many other solid cancers, lung cancer development (carcinogenesis) is a multiple step process that involves alterations of many molecular and cellular pathways regulating cancer cell proliferation, survival (anti-apoptosis) as well as neo-angiogenesis. Induction of apoptosis by chemopreventive agents can lead to the elimination of pre-neoplastic cells, affording a permanent protection against carcinogenesis.[3,4] Most anticancer therapy drugs induce apoptosis to achieve therapeutic efficacy. Novel and nontoxic agents that exert selective multi-targeting actions on cancer cell cycle progression and apoptosis as well as neo-angiogenesis will be important to combat this deadly disease through chemoprevention and innovative treatment.

Much work in the past decades has convincingly supported the obligatory need of angiogenesis for solid tumor growth and progression.[3,5–7] Hypoxia commonly develops within solid tumors because the rate of tumor cell proliferation is out-pacing the rate of blood vessel formation.[8] Hypoxia is a potent stimulator of the expression of vascular endothelial growth factor (VEGF), which is the primary mediator of angiogenesis.[8–12] The hypoxia-inducible factor (HIF-1) transactivates VEGF expression as well as several others genes such as erythropoietin, glucose transporters 1 and 3, and hexokinases.[9–11] These gene products mediate hypoxia-triggered cellular adaptive responses such as angiogenesis and glycolysis to enhance the survival of cells under hypoxic stress. In addition to VEGF, basic fibroblast growth factor (bFGF) and several other polypeptide growth factors also stimulate angiogenesis, in part through an upregulation of VEGF expression.[13,14] VEGF as well as several growth factors have been shown to be overexpressed in tumors and to play a pivotal role in the control of the proliferation and migration of endothelial cells, the essential component of a new blood vessel. Chemical agents that target endothelial proliferation, migration and capillary differentiation as well as the HIF-1/VEGF axis therefore are desirable for the angioprevention of cancer.

Naphthazarin (DHNQ, 5,8-dihydroxy-1,4-naphthoquinone) is a member of a class of antitumor agents sharing similar core structure as shikonin, adriamycin analogues and mitoxantrone.[15,16] We have earlier synthesized 6-(1-oxobutyl)-5,8-dimethoxy-1,4-naphthoquinone (OXO) from Naphthazarin[15] and reported that OXO inhibits tumor growth in mice bearing sarcoma 180 cells.[16] We have shown an inhibitory activity of OXO against tumor cell DNA topoisomerase I,[15] which would suggest a selective targeting of tumor cells active with DNA replication. These led us to hypothesize a potential use of OXO for lung cancer prevention and treatment. Because shikonin and its metabolites have been shown to inhibit angiogenesis,[17,18] in the present study, we focused on attenuation of endothelial angiogenic responses and HIF-1/VEGF axis in lung cancer cells as well as apoptosis as likely target processes for OXO to exert its anti-tumor activity.

We used a battery of *in vitro* angiogenesis tests with the well-characterized human umbilical vein endothelial cells (HUVECs) to define likely endothelial processes that are targeted by OXO. We chose the mouse LLC cell culture model to study the efficacy of OXO on hypoxia-induced changes in HIF-1α (the rate-limiting subunit of HIF-1[9]) and VEGF expression as well as for direct effects on their cell cycle and survival. In addition, LLC cells can grow rapidly in syngenic mice and the tumors are strongly dependent on angiogenesis. We have extensive experience using this model to study the *in vitro* and *in vivo* effects of novel antiangiogenic and anticancer agents from Oriental herbs.[19–21]

Here we report that OXO possesses strong antiangiogenic activities by inhibiting mitogen-stimulated endothelial cell proliferation and capillary differentiation and by downregulating HIF-1α and VEGF expression. We also show that OXO induces S phase arrest and caspase-mediated apoptosis of LLC tumor cells with greater sensitivity than in HUVECs. We report a potent *in vivo* anticancer efficacy of OXO and validation of key mechanistic biomarkers in the OXO-treated tumors.

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Material and methods

Cells and culture conditions

Culture of mouse LLC cells and human HUVECs was performed as reported in earlier publications at our laboratory.[19–22]

Cytotoxicity assay in the absence of growth factors

HUVECs or LLC cells were seeded in 100 μl of their respective complete medium on 96-well culture plates. After preincubation for 24 hr, the medium was replaced with endothelial-serum free medium without growth factors for HUVECs and serum-free RPMI-1640 media for LLC cells containing with various concentrations of OXO. The cultures were incubated for 12 hr (LLC cells) or 24 hr (HUVECs, LLC cells) and then metabolically viable cell fraction was determined by 2,3-bis[l2-methoxy-4-nitro-5-sulfo]-2H-tetrazolium-5-carboxanilide (XTT) colorimetric method,[23] which measures mitochondrial ability to metabolize the redox sensitive dye as described in a previous report.[24]

Mitogen-stimulated HUVEC proliferation assay

HUVECs (5×10^3) were seeded in complete medium onto 0.1% gelatin coated 96-well and incubated in a humidified incubator for 24 hr. The cells were deprived of angiogenic growth stimulation for 6 hr in M199 medium containing 5% FBS. Then they were treated with various concentrations of OXO in M199 containing 5% FBS, 20 ng/ml VEGF and 5 U/ml heparin or 5% FBS, 10 ng/ml bFGF and 5 U/ml heparin. After 48 hr incubation, viable cell number was estimated by XTT method as described previously.[24]

Capillary differentiation (tube formation) assay

An *in vitro* capillary differentiation assay was performed on Matrigel as originally described by Grant et al.[25] We have used this method in previous works.[19–21] In experiments where LLC-conditioned culture media were used as the source of angiogenic stimulation in the capillary tube formation assay, LLC cells were washed with phosphate-buffered saline (PBS) and then cultured in fresh RPMI 1640 medium with or without OXO, under either normoxic or hypoxic condition for 12 hr, and then the media were collected. HUVECs (5×10^4) were seeded on Matrigel in 500 μl of conditioned culture medium and evaluated as above.

RT-PCR analysis

To quantify mRNAs for HIF-1α, VEGF and β-actin (used as a control), we performed reverse transcription-polymerase chain reaction (RT-PCR) using reported primers and conditions.[26] LLC cells exposed to various concentrations of OXO under normoxic or hypoxic condition for 6 hr in RPMI-1640 supplemented with 5% serum were harvested and washed with cold PBS. Total RNA was prepared from LLC cells by using Trizol reagent (GIBCO, Life Tech, Carlsbad, CA). The RNA extraction and RT-PCR procedures have been described in our recent publication.[21]

Cell cycle analysis

Cells were stained with propidium iodide as described previously by our group.[19,20] LLC cells were treated in serum free RPMI-1640 medium for 24 hr.

Apoptotic DNA ladder and caspase activation

LLC cells were cultured in the presence or absence of OXO at various concentrations (1, 5, 10 and 20 μM) in serum free RPMI-1640 medium. Cells were collected and washed with PBS. DNA was isolated by following a published protocol.[27] The pellet was homogenized in 450 μl of lysis buffer (20 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0, 0.2% Triton X-100) by pipetting through a blue cone and incubated for 10 min on ice. The lysates were centrifuged for 15 min at 13,000*g* and the supernatants were incubated at 50°C overnight with proteinase K (2 mg/ml, Sigma). DNA was precipitated with 2 volumes of 100% ethanol and 0.1 volume of 3 M sodium acetate for 2 hr at −70°C. DNA was pelleted at 12,000*g* for 15 min and washed twice with 70% ethanol. DNA was dissolved in distilled water containing 1 mg/ml RNase A, incubated for 30 min at 37°C, and analyzed on 1.5% agarose gels.

In experiment to examine the impact of blocking caspases on the apoptosis execution induced by OXO, a general caspase inhibitor, QVD-OPH (MP-Biomedical, Aurora, OH) was added to LLC cells 1 hr before OXO treatment. Mitochondrial and cytosolic fractionations were carried out with a ApoAlert cell fraction kit (Takara Bio Company, Palo Alto, CA).

Western blotting

Total cell lysate preparation and immunobloting methods were used as we described previously.[19–21]

Measurement of secreted VEGF protein

The level of VEGF in the supernatant of LLC was measured with a commercially available ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. Briefly, LLC cells were incubated at a CO_2 level of 5% with 1% O_2 balanced with N_2 (hypoxic condition) in RPMI 1640 and treated with OXO (1, 5 μM). After 12 hr incubation, the supernatant was individually collected for ELSIA assay.

Matrigel plug angiogenesis assay in vivo

This functional angiogenesis assay was performed as originally described by Passaniti et al.[28] and in our recent publications.[19–21]

Tumor growth in vivo

LLC cells in log growth were harvested and resuspended in sterile PBS. In the first experiment as a pilot study to define the effective OXO dose range, LLC cells (5×10^5 in 100 μl PBS) were injected subcutaneously (s.c.) injected into the right flank of C57BL x DBA_2 (BDF_1) mice (Daehan Biolink Co., Chungbuk, Korea). OXO was dissolved in 1% Tween 80. One day after tumor transplantation, OXO (1.5 mg/kg, 3 mg/kg) or adriamycin (1 mg/kg) was intraperitoneally (i.p.) injected once a day for 14 consecutive days. The tumor volume was measured on day 15 after tumor inoculation with a caliper, and calculated according to the formula [(length × width^2)/2], where length represents the largest tumor diameter and width represents the smallest tumor diameter.

Based on the dose and tumor growth information of the first experiment, we repeated the experiment in C57BL/6 mice with slight modifications of dosage and treatment duration. Three days after tumor inoculation, mice received daily i.p. injection of OXO at 1 or 2 mg/kg. All mice were killed 11 days after LLC cells injection and the tumors were removed and weighed.

Immunohistochemistry

Processing and staining of tumor sections for apoptosis, angiogenesis and proliferation markers were performed as reported in our recent papers.[19–21]

Statistical analyses

Whenever appropriate and necessary, ANOVA or *t*-test (two-tailed) was used to analyze the numerical data, with $p < 0.05$ considered as significantly different.

Results

OXO inhibits VEGF-induced proliferation and tube formation of HUVECs

To define an exposure range of OXO that was without acute cytotoxicity to the quiescent endothelial cells, we measured HUVEC viability by XTT assay after 24 hr exposure in serum-free medium lacking angiogenic factor supplements (Fig. 1*b*). The serum-free condition was used to more sensitively reveal such unwanted cytotoxic effects. It is evident that exposure concentrations of ≤12 μM

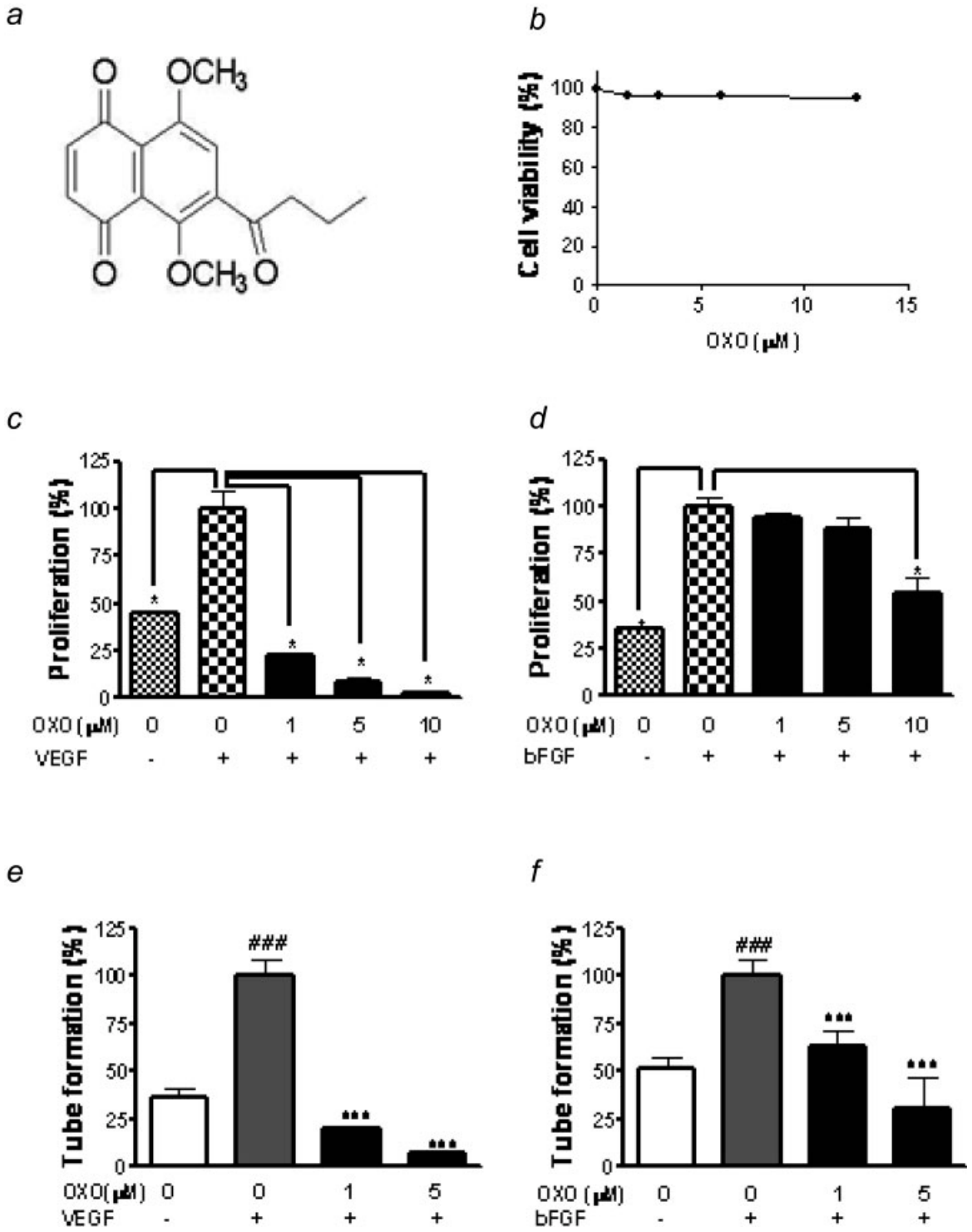

FIGURE 1 – (*a*) Chemical structure of OXO (6-(1-oxobutyl)-5,8-dimetoxy-1,4-naphthoquinone). (*b*) Effect of OXO on viability of nonangiogenically stimulated HUVECs after 24 hr exposure (serum-free medium). Viable cells were examined by XTT assay. Variability was within 3%. (*c*,*d*) Effect of OXO on (*c*) VEGF- and (*d*) bFGF-induced proliferation of HUVECs were exposed to various concentrations of OXO in M199 containing 5% FBS plus heparin in the presence or absence of VEGF 20 ng/ml or bFGF 10 ng/ml for 48 hr, and viable cell number was assessed by XTT assay. (*e*,*f*) Effect of OXO on (*e*) 5 ng/ml VEGF- and (*f*) 20 ng/ml bFGF-induced capillary tube formation on Matrigel. Data were expressed as means ± SD of triplicates. Statistical significance: ###, $p < 0.001$ *versus* unstimulated control; *, $p < 0.05$ and **, $p < 0.01$ compared with VEGF or bFGF-treated control.

were not cytotoxic to the HUVECs. No distinct morphology associated with apoptotic endothelial cells could be detected after incubation of cells on gelatin-coated culture plates for 48 hr.

Since angiogenic factor-stimulated endothelial cell proliferation is a key component of the angiogenic response,[3,6,7] we determined the effect of OXO on VEGF (20 ng/ml)- or bFGF (10 ng/ml)-

induced mitogenic response in HUVECs with a treatment duration of 48 hr. As little as 1 μM of OXO completely suppressed VEGF-induced cell proliferation (Fig. 1*c*). This inhibitory effect was not due to cytotoxicity of OXO in HUVECs, because OXO did not show any significant cytotoxic effect on nonstimulated HUVECs at 12 μM (Fig. 1*b*). OXO also inhibited bFGF-induced endothelial proliferation by greater than 50% at 10 μM, but not at lower concentrations tested (Fig. 1*d*). However, which in contrast was less effective than against VEGF-induced mitogenesis.

When seeded on Matrigel, HUVECs underwent extensive *in vitro* differentiation to form capillary-like tubes.[25] This process is promoted and sustained by VEGF and bFGF (Figs. 1*e* and 1*f*). Therefore, we tested the ability of OXO to inhibit tube formation. OXO significantly disrupted the formation of capillary-like structures in VEGF-stimulated HUVECs in a concentration-dependent manner (Fig. 1*e*). The impact of OXO on bFGF-induced tube formation was also significant even at 1 μM (Fig. 1*f*), but was less dramatic compared with that induced by VEGF.

Taken together, these data indicate that noncytotoxic levels of OXO are effective against HUVECs proliferation and *in vitro* capillary tube formation induced by either VEGF or bFGF. The response patterns suggest that OXO is more efficacious against VEGF- than bFGF-induced signaling.

OXO inhibits LLC cell growth and induces apoptosis

In contrast to HUVECs, direct exposure of LLC cells to OXO in serum-free medium caused concentration- and time-dependent decreases in viable cell number, with IC_{50} of 7 μM after 24 hr exposure (Fig. 2*a*). Cell cycle analyses of LLC cells exposed to OXO showed an increasing trend for G_2 arrest at concentrations of 5 μM or lower, and a strong S-phase arrest at a concentration of 10 μM at 24 hr (Fig. 2*b*). The flowcytometric analysis also detected an increasing sub-G1 population characteristic of apoptotic cells (Fig. 2*b*). Apoptosis was confirmed by 24 hr treatment of OXO through DNA nucleasomal fragmentation (Fig. 2*c*). It is evident that DNA laddering was increased in a concentration dependent manner.

Apoptosis execution is in general mediated by 2 caspase-activation pathways: the intrinsic mitochondria cascade (cytochrome C-caspase-9) or the extrinsic death receptor cascade (caspase-8).[29–31] Caspase-mediated cleavage of PARP, a canonical target of caspase-3, was detected at 5 μM with a concurrent decrease of full length pro-caspase-9 and the mitochondrial antiapoptosis protein Bcl-2 in the absence of a decrease of the full length pro-caspase-8 level (Fig. 2*d*), suggesting a mitochondria cascade of caspase activation for apoptosis execution.

To further confirm this prediction, we analyzed the OXO-treated LLC cells for leakage of cytochrome C from the mitochondria to the cytosol (Fig. 2*e*). OXO treatment led to a concentration-dependent increase of cytosolic cytochrome C and a corresponding depletion of mitochondrial cytochrome C. A concentration-dependent reduction of full length procaspase-9 and a reciprocal increase in the cleaved caspase-9 (active) form support the involvement of this mitochondria activation cascade. Furthermore, a general caspase inhibitor (QVD-OPH) blocked PARP cleavage, and decreased mitochondria leakage of cytochrome C and formation of cleaved caspase-9. Overall, these results indicate that OXO can exert a direct growth suppression effect on LLC tumor cells by G2/S phase arrests and by mitocondria/cytochrome C/caspase-9-mediated apoptosis at nontoxic concentrations to the HUVECs.

Effect of OXO on HIF-1α and VEGF expression in LLC cells exposed to hypoxia

Because hypoxia is a common feature of growing solid tumors and is a potent inducer of *in vivo* angiogenesis through the upregulation of HIF-1/VEGF axis,[8–12] we next examined the effect of OXO on these hypoxia-induced signaling events in LLC cells. As shown in Figure 3*a*, hypoxia increased the protein levels of HIF-1α and VEGF (lane 2 *vs*. lane 1). OXO effectively inhibited the rise in protein level of HIF-1α and VEGF in hypoxia stimulated LLC cells in a concentration-dependent fashion (lanes 3–5 *vs*. lane 2). The steady state mRNA abundance for HIF-1α and VEGF under hypoxia was decreased by OXO in a similar concentration dependent manner (lanes 7–9 *vs*. lane 6) similarly as for the respective proteins. Further experiment confirmed low basal level of the HIF-1α mRNA under normoxia condition (lane 10 *vs*. lane 12) and the suppression by OXO of the hypoxia-inducible change of this gene transcript (lane 13 *vs*. lane 12). These data support a mechanism of inhibition of the hypoxia-induction pathway by OXO is at the transcription of HIF-1α gene itself. It is noteworthy that the effective concentration of OXO to suppress hypoxia-induction of these factors in LLC cells ($IC_{50} = \sim 1$ μM) was lower than the apoptotic dose (~5 μM).

Effect of OXO on hypoxia-induced VEGF secretion in LLC cells

To verify that OXO treatment decreased the secretion of VEGF by LLC cells exposed to hypoxia, we did ELISA for the VEGF protein level in conditioned medium after 12 hr hypoxia. Indeed, VEGF was increased by hypoxia to 415 pg/ml from the normoxic level of 250 pg/ml (Fig. 3*b*). As little as 1 μM OXO completely blocked the hypoxia-induced increase of the VEGF protein level compared with the normoxic control cells (Fig. 3*b*). Functionally, OXO-hypoxia-conditioned medium was much less potent than untreated hypoxic medium to promote capillary tube formation of HUVECs when they were seeded on Matrigel (Fig. 3*c*). Together, these data indicate that OXO potently inhibits hypoxia-induction of HIF-1α and VEGF at both mRNA transcript and protein levels in the LLC cells, leading to a decreased expression and secretion of VEGF.

Effect of OXO on functional angiogenesis in matrigel plugs

The results of the above cell culture-based assays strongly supported an antiangiogenic potential of OXO. We confirmed this prediction of *in vivo* antiangiogenic activity of OXO using the Matrigel plug angiogenesis assay in the mouse. As shown in Figure 3*d*, functional angiogenesis measured by hemoglobin (Hb) content inside the Matrigel plugs was decreased by OXO by 63% and 79% of control at doses of 20 and 40 μg per plug.

OXO inhibits LLC tumor growth in mice

The strong antiangiogenic properties as well as the S/G2-arrest and caspase-mediated apoptotic actions of OXO prompted us to evaluate its safety and efficacy to inhibit the mouse LLC-tumor growth in comparison with a commonly used anti-cancer drug adriamycin in C57BL x DBA_2 mice. At a dose of 1.5 mg/kg per day, OXO suppressed tumor growth to an extent comparable or greater than 1 mg/kg adriamycin (Fig. 4*b*), without any significant changes of body weight (Fig. 4*a*). A daily injection dose of 3 mg/kg OXO inhibited tumor growth by over 90% (Fig. 4*b*). However, OXO also decreased mouse body weight, indicating possible adverse effects at this dose level (Fig. 4*a*).

Next we refined the dosage of OXO to define the safe and effective dose. A daily single i.p. injection of 1 and 2 mg OXO/kg did not affect body weight of the mice (Fig. 4*c*). Side effects, such as weight loss, hair loss, mortality and lethargy, were not observed. These nontoxic doses of OXO inhibited the kinetic of LLC tumor growth (Fig. 4*d*). By the end of experiment, the final tumor weight per mouse was decreased by 69% and 88.6%, respectively (Fig. 4*e*). These experiments firmly established the *in vivo* anticancer activity of OXO against the LLC experimental lung cancer model with a reasonable safety margin, that is, effective dosage inhibiting 50% (ED_{50}) of 0.6 mg/kg (regression analysis) *versus* a maximally tolerated dose of 2 mg/kg.

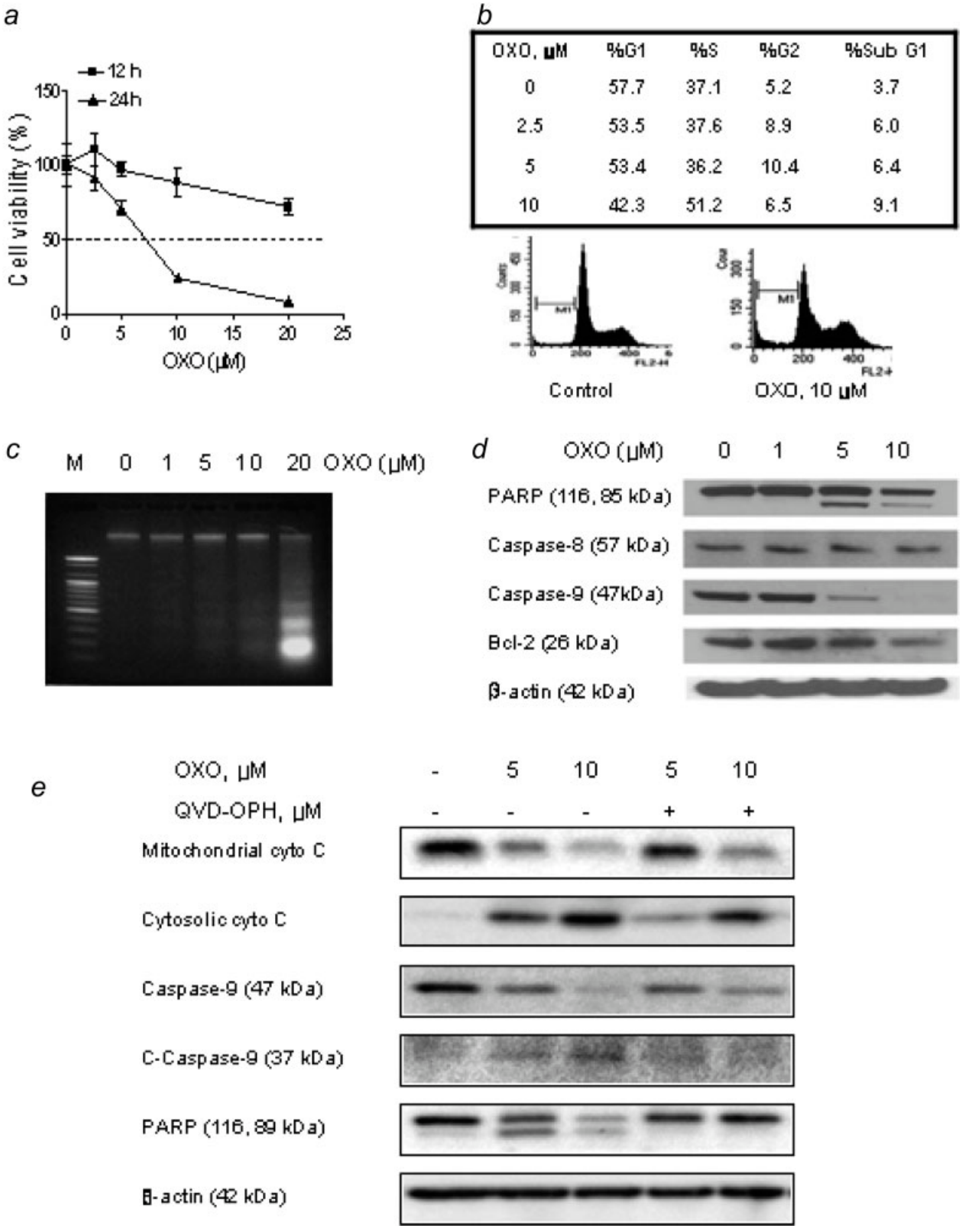

Figure 2 – Effect of OXO on cell cycle distribution and apoptosis of LLC cells. (*a*) Cytotoxicity test of LLC cells 12 or 24 hr after treatment with various concentrations of OXO in serum-free medium. The data were expressed as the mean ± SE of triplicate cultures. (*b*) Cell cycle analysis after 24 hr exposure to OXO in serum free medium. Sub-G1 fraction provides an estimate of apoptotic cells. (*c*) DNA nucleasomal fragmentation in LLC cells after 24 hr of treatment with various concentrations of OXO in serum-free medium. Genomic DNA was isolated, run on a 2% agarose gel and visualized by ethidium bromide staining. (*d*) Western blot analyses of apoptosis-related proteins in LLC cells after 24 hr of treatment with various concentrations of OXO in serum-free medium. Antibodies for caspase-8 and caspase-9 recognized the full length proenzymes. The antibody for PARP recognized both the full length and the shorter cleaved form. (*e*) Western blot analyses of the effects of OXO on cytochrome C distribution in mitochondria and cytosol and the impact of caspase inhibition on PARP cleavage and mitochondria integrity. Mitochondria and cytosol were fractionated with a ApoAlert cell fractionation kit (Takara Bio Company, Palo Alto, CA).

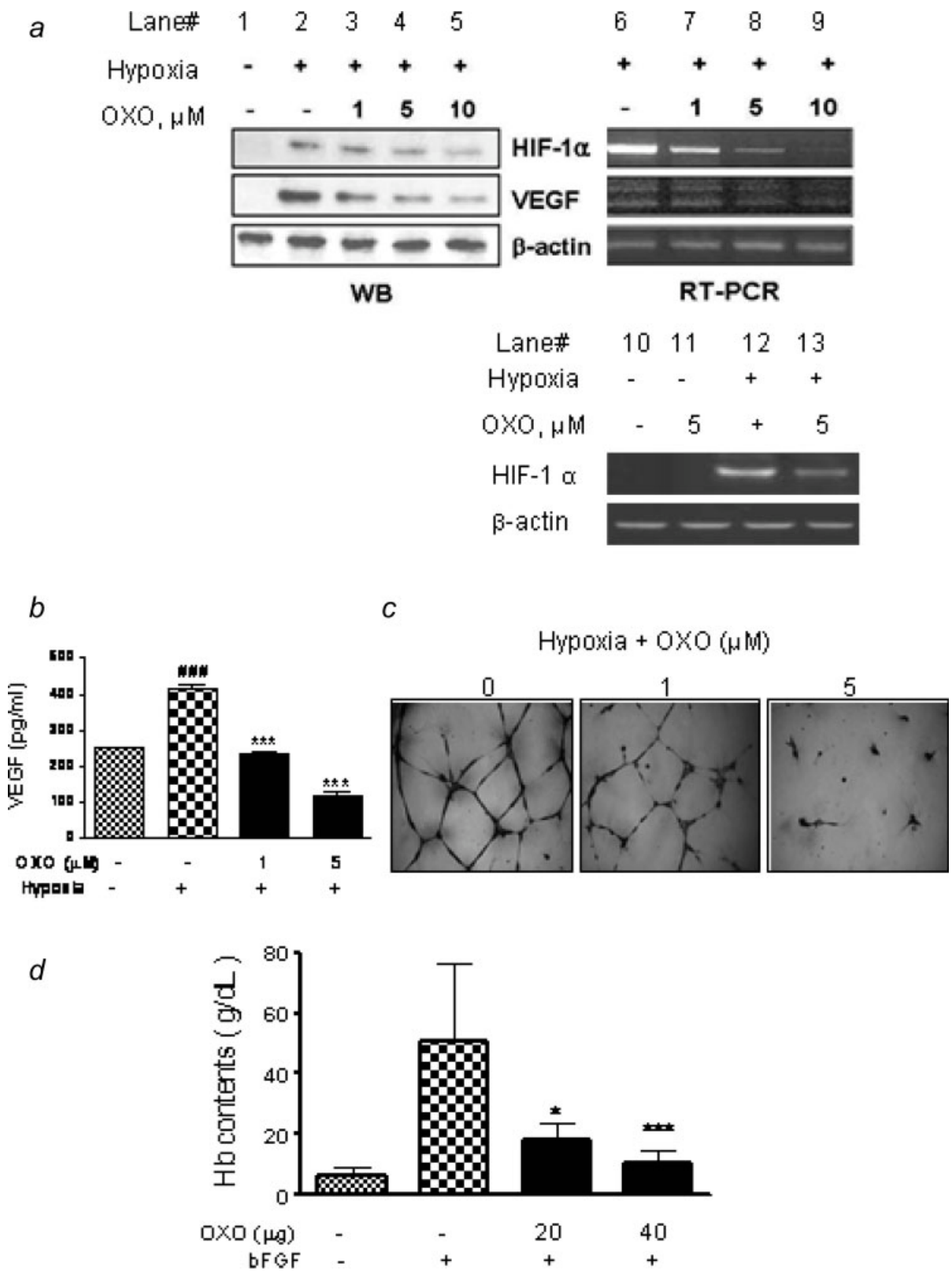

Figure 3 – (*a*) Effect of OXO on the expression of HIF-1α and VEGF in LLC cells at mRNA and protein levels. For RNA work, total RNA were isolated from LLC cells that had been treated with the indicated concentrations of OXO under 1% O_2 (hypoxia) for 6 hr. RT-PCR analysis was carried out to detect mRNA expression for VEGF, HIF-1α and β-actin. For Western blot (WB) work, LLC cells were cultured in 20% (normoxia) or 1% O_2 (hypoxia) with various concentrations with OXO for 12 hr. (*b*) Effect of OXO on hypoxia-induced VEGF secretion in LLC cells. The conditioned medium from LLC cells that had been treated with the indicated concentrations of OXO under hypoxic conditions for 12 hr was used for VEGF measurement using an ELISA kit. ###, $p < 0.001$ *versus* normoxic control; ***, $p < 0.001$ compared with hypoxia stimulated control. (*c*) *In vitro* tube formation by HUVECs exposed to conditioned media in B. (*d*) Effect of OXO on bFGF-stimulated functional angiogenesis in Matrigel plug assay in mice using hemoglobin (Hb) as an indicator. Data were expressed as means ± SD, $n = 5$. * and ***, $p < 0.05$ and $p < 0.001$, respectively, compared with bFGF-stimulated control.

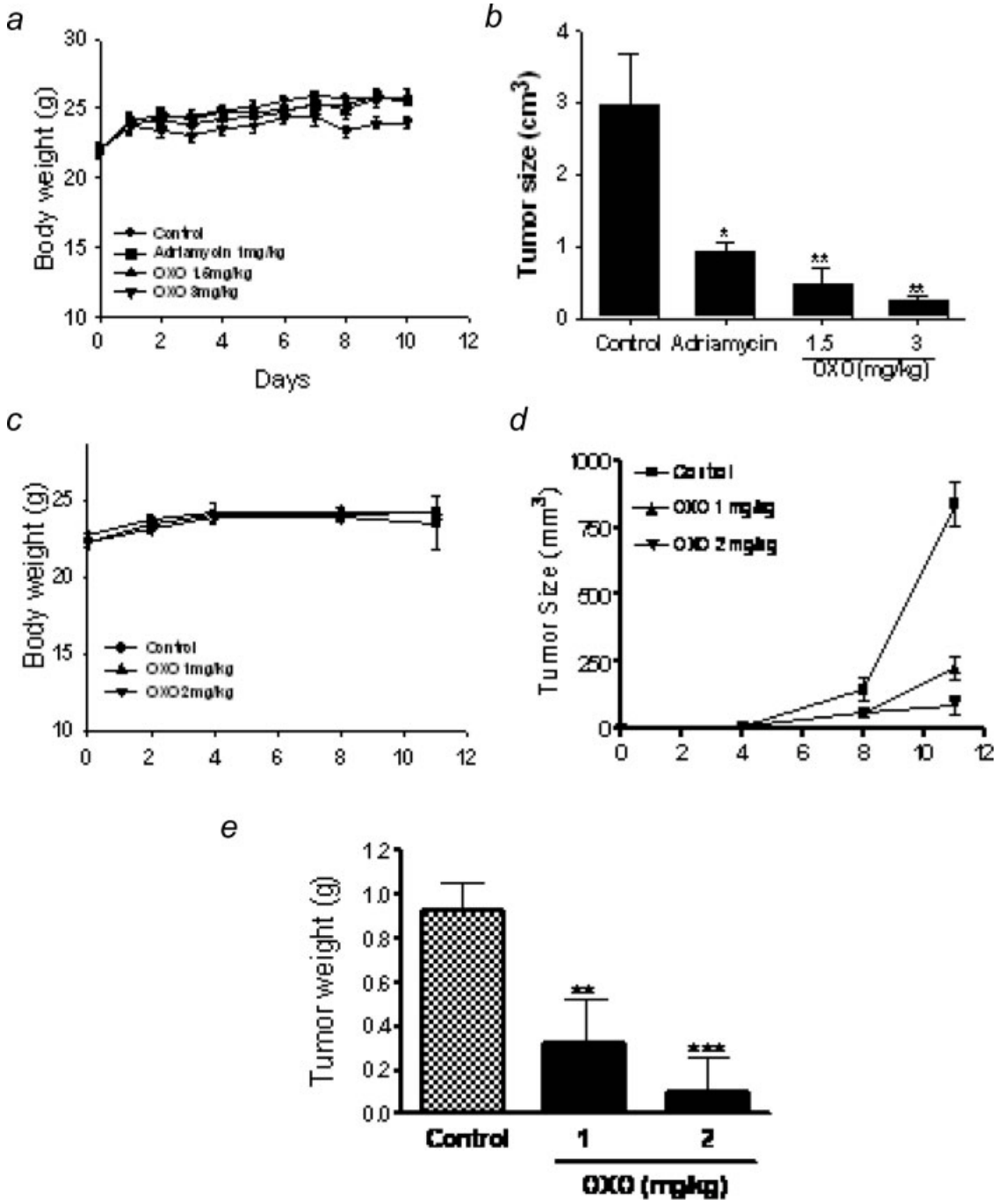

FIGURE 4 – (*a*, *b*) Experiment 1: Effect of OXO on the body weight (*a*) and tumor growth of inoculated LLC cells (*b*) in C57BL x DBA_2 mice. All mice were implanted by SC injection with LLC cells (5×10^5/100 µl PBS) on the right flank. Three days later, mice received daily i. p. injection of solvent vehicle (control), OXO or adriamycin. Panel (*b*) shows calculated tumor volume at necropsy 15 days after tumor inoculation. Data are presented as means ± SE; $n = 5$. * $p < 0.05$ and ** $p < 0.01$ compared with control. (*c*–*e*) Experiment 2: Effect of OXO on the body weight (*c*), tumor growth kinetics (*d*) and final tumor weight at necropsy 11 days after LLC cell inoculation (*e*) in C57/BL6 mice. Data were presented as means ± SE; $n = 5$. ** $p < 0.01$ and *** $p < 0.001$ compared with control.

Effect of OXO on tumor apoptosis and cell proliferation biomarkers in the LLC tumor model

To validate the involvement of inhibition of tumor cell proliferation and cell death in the anticancer action of OXO, we examined the tumor sections by terminal deoxynucleotidyl transferase-mediated dUTP-nick end labeling (TUNEL) assay as a marker for apoptosis and for changes in the expression level of a proliferation marker proliferating cell nuclear antigen (PCNA) (Fig. 5*b*). OXO significantly increased the number of TUNEL positive apoptotic cells in comparison with untreated control (Fig. 5*c*). OXO also significantly decreased the percentage of tumor epithelial cells positive for PCNA staining (Fig. 5*d*). These data support the inhi-

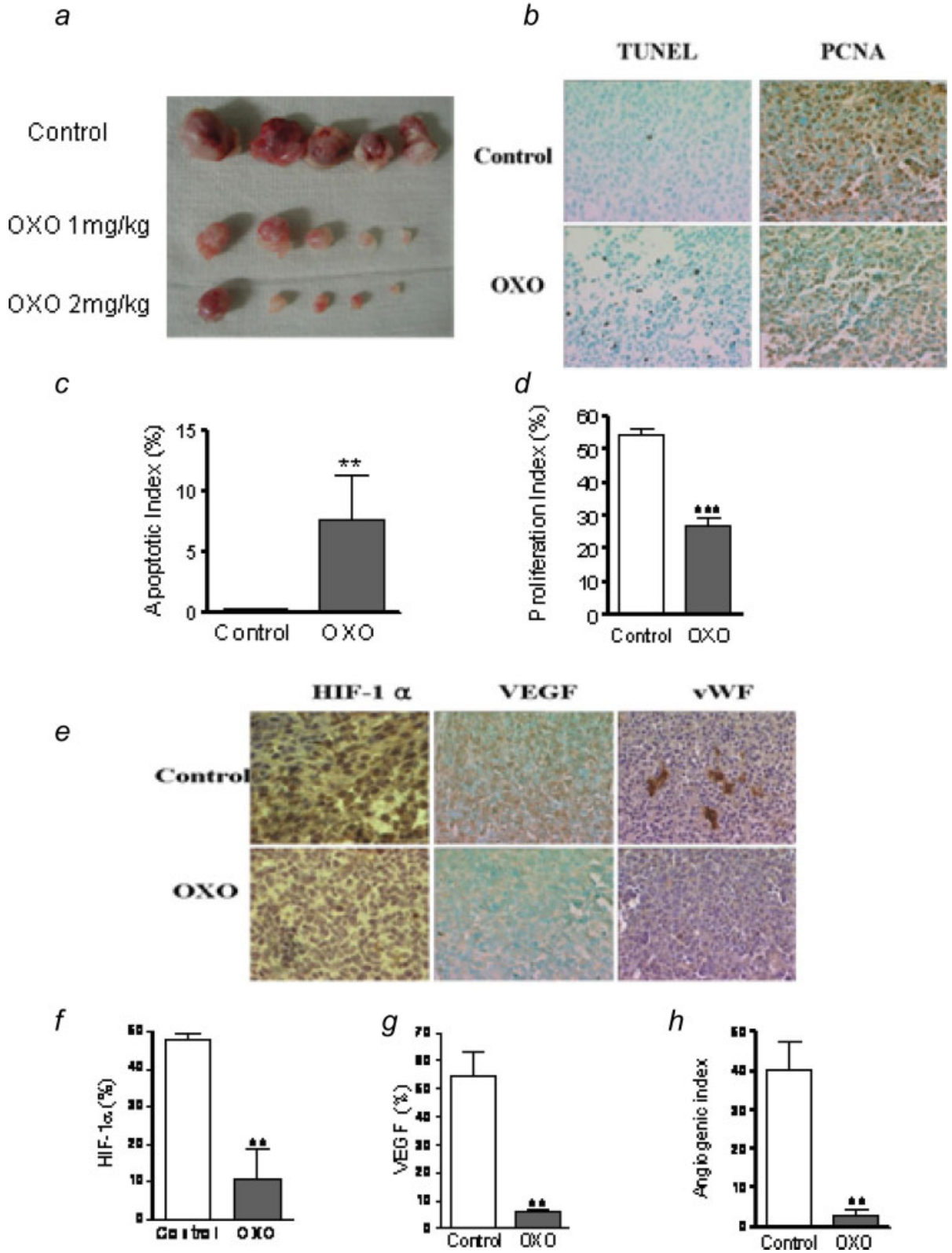

FIGURE 5 – (*a*) Photograph of LLC tumors from each group of C57/BL6 mice from the second experiment. (*b*) Representative immunohistochmical staining for TUNEL (column 1) and PCNA (column 2) of tumors from control and OXO (2 mg/kg)-treated groups. The TUNEL apoptotic (*c*) and PCNA proliferative (*d*) indices were calculated as the ratio of positive cells to total stained cells in tumor sections. Values represent means ± SD; $n = 5$. ** $p < 0.01$ and *** $p < 0.001$ compared with control. (*e*) Representative immunohistochemical staining for HIF-1 α (left column), VEGF (middle column) and vWF (right column) of tumors from control and OXO (2 mg/kg)-treated groups. Quantitation of each index was presented in the graphs: HIF-1 α (*f*), VEGF (*g*) and vWF (*h*). Values represent means ± SD; $n = 5$. ** $p < 0.01$ and *** $p < 0.001$ compared with control.

bition of tumor cell proliferation and induction of apoptosis contribute to the *in vivo* anticancer activity of OXO.

OXO decreased HIF-1α, VEGF and microvessel density in tumors

To validate the targeting of HIF-1α and VEGF axis of angiogenesis signaling by OXO *in vivo*, we assessed HIF-1α and VEGF protein expression in tumor sections from control and OXO treated mice by immunohistochemistry staining (Fig. 5*e*). As expected, the HIF-1α was located in the nucleus of tumor cells (Fig. 5*e*). Being a secretory protein, VEGF was observed mostly in the cytoplasm (Fig. 5*e*). Tumors from OXO-treated mice contained much decreased levels of immunostainable HIF-1α (Fig. 5*f*) and VEGF proteins (Fig. 5*g*). Furthermore, the intratumoral microvessels detected by staining for von Willibrand factor (vWF)/factor VIII (Fig. 5*e*) were significantly fewer in the OXO-treated tumors (Fig. 5*h*). These data suggest the involvement of antiangiogenesis for the *in vivo* anticancer activity of OXO.

Discussion

The obligatory dependence of solid tumor growth and progression on angiogenesis has now been well supported and accepted.[3,5–7] Tumor angiogenesis involves complex and multiple vascular endothelial as well as tumor epithelial processes. The endothelial processes include angiogenic factor-stimulated changes in the functions of the vascular endothelial permeability, degradation of the basement membrane by metalloproteases, migration, remodeling and proliferation of endothelial cells to form capillary tubes, to name a few. On the tumor epithelial cell side, the most important process is probably the increased production of VEGF due to hypoxia and other autocrine or paracrine growth factors.[8–14] Interruptions of 1 or more steps in such processes can negatively affect angiogenesis and inhibit early lesion growth to lower the cancer risk by angioprevention.

In the present study, we provided *in vitro* data supporting the antiangiogenic potential of OXO *via* targeting a number of molecular mediators and processes in vascular endothelial cells. We showed that at concentrations that did not inflict cytotoxicity to the nonstimulated HUVECs (Fig. 1*b*), OXO strongly inhibited VEGF-stimulated proliferation (Fig. 1*c*) and disrupted their ability to form capillary tubes under VEGF stimulation on Matrigel (Fig. 1*e*). OXO also inhibited these cellular responses induced by bFGF (Figs. 1*d* and 1*f*), but required a greater concentration, supporting a level of OXO specificity toward VEGF signaling (Figs. 1*c* and 1*e* *vs*. Figs. 1*d* and 1*f*). The detailed signaling pathways underlying these differential responses require further investigation.

Another novel finding is the identification of HIF-1α as a target for OXO to suppress the production of VEGF by the LLC cells (Fig. 3*a*). Our data revealed that, under hypoxic condition, OXO effectively downregulated the expressions of HIF-1α at both mRNA and protein levels in LLC cells (3A), leading to reduced VEGF expression and secretion (Fig. 3*b*). Functionally, concentrated conditioned medium (CM) prepared from OXO-treated hypoxic LLC cells was less able to support sustained tube formation of HUVEC on Matrigel (Fig. 3*c*). These effects were observed at OXO concentrations much lower than the apoptotic effect in LLC cells (Fig. 2). Consistent with the antiangiogenic action of OXO based on the *in vitro* tests, we validated the suppression of HIF-1 and VEGF expression in LLC tumors grown in syngenic mice *in vivo* (Fig. 5).

In addition to the antiendothelial responses above, OXO also induced G2/S-phase arrests (Fig. 2*b*) and apoptosis of LLC cells (Fig. 2*c*–2*e*) at concentrations that are not cytotoxic to HUVECs. The G2-phase arrest action of lower levels of OXO (5 μM or lower) of the LLC cells could be in agreement with the DNA topoisomerase I inhibitor action of OXO[15] because S-phase cells actively replicating DNA would be most susceptible to blockage of DNA unwinding, resulting in a slowing down of cells through G2. The S-phase-arrest action of OXO at a concentration that resulted in significant death could a reflection of inability of the dying cells to move out of S-phase.

In terms of the caspase-activation signaling, available data suggest the mitochondrial pathway is the main target for OXO as indicated by the activation of caspase-9, the decrease of mitochondria protective protein bcl-2 and the lack of cleavage of full length pro-caspase-8 (Fig. 2*d*), which is the apical caspase for the death receptor pathway.[29–31] This hypothesis was further supported by the increased release of cytochrome C from mitochondria to cytosol in the OXO-treated LLC cells (Fig. 2*e*). Our data based on caspase inhibitor study affirm the crucial role of caspase activation for OXO-induced PARP cleavage and suggest a possible feedback loop from downstream caspases to amplify the mitochondria targeting action of OXO to release cytochrome C. Further work is needed to elucidate the detailed mechanisms by which OXO results in cytochrome C release and caspase-activation cascade. Nevertheless, our data suggest a selective apoptosis-inducing effect of OXO against neoplastic cells in comparison with the normal HUVECs. Extending on the cell culture work, we established that OXO potently inhibited LLC tumor growth with a reasonable safety margin as judged by the lack of suppression effect on body weight (Fig. 4) accompanied by increased tumor cell apoptosis and decreased proliferating cells (Fig. 5).

It remains to be determined whether antiangiogenesis is the primary event leading to a secondary suppression of tumor cell proliferation and induction of apoptosis or *vice versa*. A carefully designed time course study focusing on the early responses in the OXO-treated tumors should help to shed light on the temporal and cause-effect relationship among these processes. It would also be interesting to determine what happens to the treated tumors if the OXO treatment is stopped.

In summary, our results suggest that the anticancer activities of OXO are mediated by the potent inhibition of hypoxia-induction of HIF-1/VEGF axis and angiogenesis and by G2/S-phase arrests and caspase-mediated apoptosis of tumor cells. Testing of the efficacy of OXO through oral administration (drinking water, diet) in relevant primary lung carcinogenesis models will help to evaluate the merit of this novel antiangiogenic and apoptotic agent for the practical chemoprevention of lung cancer. In addition to cancer chemoprevention and treatment, OXO may be applicable to treating other angiogenesis-dependent pathologic conditions.

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