Carbon Monoxide Expedes Metabolic Exhaustion to Inhibit Tumor Growth

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Abstract

One classical feature of cancer cells is their metabolic acquisition of a highly glycolytic phenotype. Carbon monoxide (CO), one of the products of the cytoprotective molecule heme oxygenase-1 (HO-1) in cancer cells, has been implicated in carcinogenesis and therapeutic resistance. However, the functional contributions of CO and HO-1 to these processes are poorly defined. In human prostate cancers, we found that HO-1 was nuclear localized in malignant cells, with low enzymatic activity in moderately differentiated tumors correlating with relatively worse clinical outcomes. Exposure to CO sensitized prostate cancer cells but not normal cells to chemotherapy, with growth arrest and apoptosis induced in vivo in part through mitotic catastrophe. CO targeted mitochondria activity in cancer cells as evidenced by higher oxygen consumption, free radical generation, and mitochondrial collapse. Collectively, our findings indicated that CO transiently induces an anti-Warburg effect by rapidly fueling cancer cell bioenergetics, ultimately resulting in metabolic exhaustion. Cancer Res; 73(23); 7009-21. ©2013 AACR.

Introduction

Epithelial cancers, including prostate, breast, and lung cancer, are still leading causes of deaths in the United States and treatment for advanced disease is limited (1). A standard of first-line care for advanced and metastatic cancers remains chemotherapy such as taxols, doxorubicin, and cisplatin (2). Rapid proliferation of primary tumor and cancer cell survival during spread to distant organs as well as resistance to treatment are possible in part due to the remarkable metabolic adaptation known as the Warburg effect (3). The Warburg effect is characterized by increased glucose uptake and elevated glycolysis with a limited oxygen consumption rate (OCR) resulting in lactic acid fermentation (4). High rates of energy consuming processes including protein, DNA, and fatty acid synthesis in cancer cells is often accompanied by an increased oxidative state of dysfunctional mitochondria (5). The promotion of tumor growth requires, in part, a selection of cancer cells with repressed mitochondrial activity and biogenesis (6). Defects in mitochondrial ROS metabolism from electron transport chains in cancer cells have been linked directly to increased cancer cell glucose metabolism. The free radical theory of cancer implicates ROS as a principal cause of early mutations as well as being involved in the response to treatment (7–11).

Heme oxygenases (HO), which degrade heme to biliverdin, carbon monoxide (CO), and iron, are critical modulators of metabolism and mitochondrial activity. Expression of HO-1, the stress-inducible isofrom, is strictly regulated, whereas HO-2 is ubiquitously expressed primarily in brain and testes. Their functional role in cancer has not been clearly elucidated and remains controversial. HO-1 can impart potent antiproliferative and proapoptotic effects via antioxidant mechanisms as shown in breast and lung cancer cell lines (12, 13). Better survival rates were observed in patients with colorectal cancer where HO-1 expression correlated with lower rates of lymphatic tumor invasion. In contrast, overexpression of HO-1 has been shown to accelerate pancreatic cancer aggressiveness by increasing tumor growth, angiogenesis, and metastasis (14). Similar effects were observed in melanoma (15), gastric (16), and renal cancers (17). In patients with prostate cancer, HO-1 is localized in the nucleus and correlated with cancer progression (18). Nuclear HO-1 was also detected in head and neck squamous carcinomas and associated with tumor progression (19). Recently, nuclear HO-1 has been linked to resistance to imatinib in chronic myeloid leukemia (20). Further evidence for HO-1 in cancer incidence presides in the identification of a GT length polymorphism of the HO-1 promoter that is highly correlated with cancer severity (21). Individuals with long GT repeats in the HO-1 promoter and associated low expression of
HO-1 showed a higher frequency of gastric or lung adenocarcinoma and oral squamous cancer versus those with short GT repeats and higher HO-1 expression (22). CO, biliverdin, bilirubin as well as iron and ferritin serve as potential modulators of tumorigenesis however all have been minimally studied in cancer (23).

In the present studies, we first conducted a detailed analysis of a large cohort of patients with prostate cancer and confirmed HO-1 nuclear localization in moderately advanced tumors where it is enzymatically inactive and therefore may be a critical regulator of cancer progression. We tested the hypothesis that HO-1, through its ability to generate CO, modulates cancer cell growth in vitro and in vivo using human and murine prostate and lung cancer models. Paradoxically, CO rapidly enhanced mitochondria activity of cancer cells that results in metabolic exhaustion and cellular collapse causing tumor regression. Furthermore, CO increased cancer cell sensitivity to chemotherapeutics 1,000-fold while simultaneously protecting normal cell growth and viability.

Materials and Methods

Prostate cancer samples and tissue microarray

Benign and malignant samples of 482 patients undergoing radical prostatectomy for localized prostate cancer were subjected in duplicate to tissue microarray (TMA) constructs of 1.0 mm in diameter and scored for immunohistochemical staining intensity as previously described (24). The majority of samples were successfully prepared (~95%), and Gleason grades were evaluated by a National Board–certified pathologist (L. Helczynski) in the prostate cancer specimens from 351 before preparation of TMA. The group of samples consisted of 246 samples with Gleason grade 3 and 105 samples with Gleason grades 4 and 5. The study was approved by the Ethics Committee, Lund University and the Helsinki Declaration of Human Rights was strictly observed.

Immunohistochemistry

Immunohistochemical staining of paraffin-embedded sections from human TMA blocks was conducted as previously described (25). Mouse antibody against HO-1 (Stressgen, #OSA-110) was used at 1:200 dilution. Secondary antibody was used as a negative control. Immunohistochemistry, immunofluorescence, and immunostaining on the cell lines and mouse tumor samples were conducted as previously described (26). Briefly, formalin-fixed or Zn-fixed paraffin-embedded tumor tissues were processed for antigen retrieval with high-pressure cooking in citrate buffer for 1 hour. Tissues sections were then blocked with 7% horse serum for 30 minutes, followed by incubation with primary antibody overnight. Biotin-labeled secondary antibody (Vector Laboratories) was applied for 1 hour at room temperature followed by Vectastain Elite ABC kit and detection with ImmPact DAB (Vector Laboratories). Immunofluorescence staining was conducted on frozen tumor sections or cultured cells on glass coverslips. Tissues or cells were fixed with 2% paraformaldehyde for 10 minutes followed by permeabilization with 0.05% Triton X-100. After blocking with 7% horse serum, sections were probed with primary antibody and secondary fluorescently labeled antibody (Molecular Probes). Hoechst-33258 (Molecular Probes, Invitrogen) was used to stain the nuclei.

The following antibodies were applied: polyclonal rabbit anti-HO-1 (Stressgen) was used at 1:1,000. Prohibitin antibody was purchased from Epitomics Inc. Cytochrome c antibody was obtained from Cell Signaling, mtTFA and vimentin antibodies were purchased from Santa Cruz Biotechnology. Anti-mouse Ki-67 antibody was from Dako. Anti-mouse Thy-1, CD31 antibody was from BD Biosciences, Pharmingen and anti-P (Ser10)-histone H3 was from Cell Signaling. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was conducted using ApopTag Peroxidase In Situ Apoptosis Detection Kit (Chemicon, Millipore) following manufacturer’s protocol.

Animal tumor models

The tumor models were approved by the BIDMC IACUC. Nai/nu mice were purchased from Taconic at 7 weeks of age. Mice were provided food and water ad libitum as needed. After 1-week acclimatization, 1 × 10⁶ PC3 cells were injected into the right flank of mice. Tumors were established for 2 weeks, at which point treatment with CO ± doxorubicin was begun. Mice were exposed to CO (250 ppm, 1 h/d) as previously described (27). Doxorubicin (8 mg/kg) was given i.v. twice per week just before CO/air exposure. Tumor volume (0.52 × width × length) was measured and calculated every day for 14 days. After 14 days, mice were sacrificed and tumors were harvested. In the orthotopic model of PC3 xenografts, 1 × 10⁶ PC3 cells were injected to the right lobe of prostate under anesthesia. Tumors (n = 4 per group) were established for 2 weeks followed by CO/air treatment for 4 weeks. CO was administered every day for 1 hour at 250 ppm. TRAMP mice (Jackson Laboratories) were treated with CO (250 ppm/d, 5 d/wk) starting at 5 weeks of age. Treatment was continued for 20 weeks. Tumors were harvested and processed for immunohistochemical analysis thereafter. Kras4bG12D transgenic mice were kindly provided by Dr. Varmus and were on doxycycline chow at 5 weeks of age and CO was started after 8 weeks of doxycycline treatment. CO (250 ppm, 5 d/wk) was continued for 5 weeks and the lungs were harvested for histology.

Cell culture and treatment

The human prostatic cell line, PC3, human breast cancer cells (MDA-468 and T47D), hepatocarcinoma (HepG2), cervical cancer cells (HeLa), skin fibroblasts (NIH3T3), and lung adenocarcinoma cell line (A549) were purchased from American Type Culture Collection or provided by Dr. Balk and Dr. Chen (BIDMC) and were cultured in RPMI or Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS and antibiotics. Human bronchial epithelial cells were purchased from American Type Culture Collection (ATCC) and cultured following manufacturer’s protocol. PNT1A were kindly gifted by Dr. Nishtman Dizeyi (Lund University, Malmo, Sweden) and were cultured in RPMI medium supplemented with 10% FBS and antibiotics. A549 rho(o) cells that are deprived of mitochondria were generated after 3 weeks of culture in medium containing ethidium bromide (20 μg/mL). Mouse embryonic...
fibroblasts from p53+/+ and p53−/− mice were kindly gifted by Dr. Tyler Jacks (MIT, Boston, MA) and were cultured in DMEM with 10% FBS and antibiotics. Cells were treated with camptothecin (0.001–10 μg/mL Sigma) or doxorubicin (10 μg/mL Sigma) and vehicle control (dimethyl sulfoxide) for 1 to 24 hours. Cotreatment with CO (250 ppm) was conducted as previously described (27). Pegylated catalase (3000 U/mL) and SOD (10 U/mL) were from Sigma and were used 1 hour before CO treatment.

**Transient transfection**

PC3 cells were transfected using Amaxa nucleasection or Lipofectamine 2000 as previously described (24). pCMV control vector or human HO-1 cDNA encoding vector were used for transfection. Transfection efficiency was between 40% and 50% as estimated with overexpression of GFP by fluorescence microscopy.

**Comet assay**

PC3 cells were treated with CO or air for 24 hours and harvested for COMET SCGE Assay (Assay Designs). The manufacturer’s protocol was followed. Cells were viewed by Zeiss Axiovert Apotome Fluorescent microscope at ×40. At least 100 cells were counted, and the amount of comets was calculated and expressed per 100 cells.

**Immunoblotting**

Cells were lysed by brief sonication in ice-cold lysis buffer [150 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.5, 1% NP-40, 10 mmol/L NaF, 1% SDS, 1 mmol/L EDTA, pH 8.0, 10 mmol/L phenylmethylsulfonyl fluoride (PMSF)] and the protease inhibitor cocktail Complete Mini (Roche). Samples were centrifuged for 20 minutes at 14,000 × g at 4°C and clear supernatants were collected. Protein concentrations were measured using a BCA Protein Assay Kit (Pierce). Twenty to 30 micrograms of each protein sample was electrophoresed on a NuPAGE 4–12% Bis-Tris Gel (Invitrogen) followed by transfer to polyvinylidene difluoride (PVDF) membrane (Ready Gel Blotting Sandwiches; BioRad). The membranes were blocked with 5% nonfat dry milk, probed with appropriate primary antibodies at concentration of 1:1,000 followed by horseradish peroxidase-conjugated secondary antibodies at a dilution of 1:5,000, and visualized using Super Signal West Pico chemiluminescent substrate (Pierce) or Femto Maximum Sensitivity substrate (Pierce), followed by exposure to the autoradiography film (ISC BioExpress). The following antibodies were applied: rabbit anti-caspase-3; rabbit anti-cleaved caspase-3 (Cell Signaling Technologies); goat anti-Gadd45 (Santa Cruz Biotechnology); rabbit anti-heme oxygenase-1 (RDI-Fitzgerald Industries Int); mouse anti-β-actin (Sigma-Aldrich); mouse anti-GAPDH (Calbiochem); and rabbit anti-cyclin A2 (Santa Cruz Biotechnology).

**HO-1 activity and CO levels in the tissues**

PC3 cells were treated with 10 μg/mL doxorubicin or camptothecin for 4 hours, and nuclear/cytoplasmic fractions were isolated. HO-1 activity was measured as previously described (28). We assume that only HO-1 was contributing to the changes in HO activity assay as HO-2 remained unchanged in response to doxorubicin and camptothecin treatment for 4 hours (data not shown). Measurements of CO concentrations were conducted on tissue sections as described by Verma and colleagues (29). Tumor and normal tissues were harvested immediately after the last exposure of mice to CO and tissues were processed for gas chromatography as described. Briefly, CO was liberated as a gas in a sealed glass vial by adding 25 μL of water and 5 μL of sulfosalicylic acid [SSA; 30% (wt/vol)] to 30 μL of diluted tissue homogenate. The vials were incubated on ice for 10 minutes before being analyzed. The gas in the headspace of the vials was then analyzed quantitatively with a gas chromatograph equipped with a reducing-compound photomultiplier detector (Peak Laboratories), which allowed the CO in gas to be quantified to concentrations as low as 1 to 2 parts per billion. The amount of CO was then calculated using a calibration curve prepared from CO standards.

**RNA isolation and real-time PCR**

Total RNA was isolated from PC3 cells after treatment with 10 μg/mL camptothecin with or without CO for 6 or 24 hours using RNeasy Kit (Qiagen, Inc). Two micrograms of RNA was used for cDNA synthesis (Invitrogen). The region of Gadd45α was amplified with specific primers: F-5′ TGCTCAGCAAACCTGAGT3′; R-5′GCAGGCACACACACAGTGA3′. The following primers to β-actin were used: F-5′ CGCGAGAGAAGGAGCCGAC3′; R-5′ TCACCGGGATCCATCAGA3′. Real-time PCR reactions were conducted using 1×SYBR Green PCR Master Mix (BioRad). Primers for MMP2, urokinase plasminogen activator (uPA), and VEGF were previously described (24).

**Apoptosis, viability, and clonogenic assays**

PC3 cells were seeded at 1 × 10^5 concentration in 6-well plates 24 hours before experiment. Cells were treated with camptothecin (0.001–10 μg/mL) or doxorubicin (10 μg/mL) with CO/air for 24 hours. Cells were harvested and apoptosis rate was measured using Annexin V-FITC staining according to the manufacturer’s protocol (BD Biosciences). To test cell viability, PC3 cells were stained with crystal violet solution (Sigma-Aldrich) for 10 minutes, followed by extensive wash with double distilled water. Cells were dried and dissolved in 10% acetic acid, followed by measurement of absorbance at 560 nm on the ELISA reader.

**Clonogenic assay.** Cells were treated in the presence or absence of CO ± chemotherapeutics as above for 24 hours. After 24 hours, cells were trypsinized and seeded as a subculture with dilution of 1:10 to 1:100. Colony counts were conducted 2 weeks after seeding.

**Cell fractionation**

Nuclear and cytoplasmic fraction of PC3 cells were conducted using the Subcellular Fractionation Kit following the manufacturer’s protocol (Pierce) or BioVision Nuclear/Cytoplasmic Kit extraction (BioVision). Briefly, cells were treated as described above, washed twice with PBS, and trypsinized. Pellets were collected and resuspended in ice-cold cytoplasmic extraction reagent (CERI) buffer and vortexed. Lysates were incubated on ice for 10 minutes, and CERII buffer was added.
and lysates were incubated for additional 1 minute on ice. After vortexing, lysates were centrifuge at 16,000 \( \times g \) and supernatants were collected for the cytoplasmic fraction. Pellets were resuspended in ice-cold nuclear extraction reagent (NER) buffer and incubated on ice for 30 minutes. Nuclear and cytoplasmic fractions were analyzed by immunoblotting as described above.

**Mitochondrial activity and metabolic profiling**

**SPECT.** We used technetium-99m methoxyisobutyl isonitrile (\(^{99m}\)Tc-sestamibi) single-photon emission computed tomography (SPECT) in PC3 cells treated with CO (250 ppm) or CORM-A1 (20 or 100 \( \mu \)mol/L) for 24 hours in vitro. Technetium labeling was conducted at BIDM Imaging Facility, and the radiation intensity was evaluated that corresponded to the mitochondrial activity.

** Seahorse experiments**

Cells were seeded in 24-well plate (3 \( \times 10^4 \)) 24 hours before experiment and treated with CO (250 ppm) or air for 30 minutes or CO was bubbled into the medium and transferred to the reader immediately prior measurment. OCR and extracellular acidification rate (ECAR) were measured over time.

**LDH and glucose measurements**

PC3 cells were treated for 6 hours with CO (250 ppm) and harvested in the manufacturer’s lysis buffers to measure glucose or lactate (Biovision, Glucose or Lactate Kits) and process for colorimetric analysis of metabolites.

**MitoTracker Red**

CMCRos was purchased from Invitrogen, Molecular Probes and was used at 0.2 \( \mu \)mol/L concentration. Cells were treated for 3.5 hours with doxorubicin (10 ng/mL) \( \pm \) CO and MitoTracker Red CMCRos was added for the last 30 minutes of treatment. Cells were washed and fixed with 2% paraformaldehyde followed by staining with Hoechst.

**Statistical analyses**

Statistical analyses were conducted using SPSS version 13.0 (SPSS Inc.). The differences in the expression of different markers between benign and cancer specimens were evaluated by paired Wilcoxon rank-sum test or Kruskal–Wallis test. Distributions of overall survival and disease-free survival were estimated by the method of Kaplan–Meier, with 95% confidence intervals. Differences between survival curve were calculated using the log-rank test. Data are presented as the mean \( \pm \) SD and are representative for at least 3 independent experiments. Student t, ANOVA, and Wilcoxon tests were used for estimation of statistical significance for the experiments (\( P < 0.05 \)).

**Results**

**Nuclear HO-1 with low activity in prostate cancer correlates with poorer prognosis**

HO-1 has been shown to be expressed and function in the nucleus as a regulator of oxidative stress response transcription factor at the expense, however, of losing enzymatic activity (30). On the basis of these data, we hypothesized that nuclear translocation of HO-1 influences the response of cancer cells to therapy. We evaluated 482 tumor specimens from patients with prostate cancer with corresponding benign tissues by tissue microarray to assess the correlation between nuclear HO-1 expression and clinical tumor characteristics. The majority of benign samples showed low overall expression of HO-1 (Fig. 1A–B and Supplementary Fig. S1A). In contrast, Gleason grade 3 specimens showed significantly higher expression of HO-1 in the nucleus than that of corresponding benign tissue (Fig. 1C and D; \( \ast \), \( P = 0.047 \)). The intensity of nuclear HO-1 staining declined in Gleason grade 4–5 specimens as compared with Gleason grade 3 but did not achieve statistical significance.
(Fig. 1A–H). Finally, there was no significant difference in cytoplasmic staining of HO-1 in prostate cancer versus adjacent benign samples (data not shown). We reasoned that high nuclear HO-1 expression in moderately differentiated tumors may correspond to the level of oxidative stress or hypoxia that drives progression of differentiated tumors to more advanced disease.

We next evaluated the clinical significance of nuclear HO-1 expression by correlating HO-1 staining with biochemical recurrence (BCR) of prostate cancer as measured by prostate-specific antigen in the circulation (31) as well as overall patient survival (Fig. 1H). High nuclear HO-1 (arbitrary intensity = 3) expression in the cancer biopsies in comparison to low (arbitrary intensity = 1) nuclear HO-1 predicts a shorter time to BCR (Fig. 1F; \( P = 0.007 \)). After a 20- to 30-month follow-up period, a 20% greater number of patients with high nuclear HO-1 relapsed in comparison to patients with low nuclear HO-1 (\( P = 0.046 \)), indicating a shorter time to recurrent disease in patients with prostate cancer with a lack of enzymatically active nuclear HO-1.

**HO-1 activity and CO influence the cancer cell response to genotoxins**

Nuclear localized HO-1 is a hallmark of cancer progression in prostate cancer (18). Comparing the HO-1 localization profiles and activity of noncancerous cells (epithelial cells and fibroblasts) to cancerous cells (PC3, A549, HepG2, T47D, MDA-468, HeLa), we found that cancer cell lines showed high nuclear expression and low cytoplasmic expression, whereas noncancerous cells showed high cytosolic expression and low nuclear expression (Fig. 2A and B). We show that when HO-1 is localized to the nucleus it loses its activity in response to common chemotherapeutics (Fig. 2C; ref. 30). On the basis of nuclear localization of HO-1 with low activity (30), we next asked whether HO-1 is important in mediating the effectiveness of chemotherapeutic treatment in vitro. DNA damage causes mutations and carcinogenesis as well as induces cancer cell death. HO-1 expression in normal cells is required for appropriate DNA repair in response to chemotherapy or irradiation (32). Using camptothecin and doxorubicin to induce DNA damage, we tested the role of HO-1 and CO on cancer and normal cell death. We observed significant cell death with camptothecin or doxorubicin in vector control–treated PC3 cells as expected. Overexpression of HO-1 induced further death of PC3 cells in response to camptothecin or doxorubicin (Fig. 2D). A similar effect was observed in cells after exposure to a low concentration of CO (Fig. 2 and Supplementary Fig. S1B and S1C; ref. 33). Dose escalation experiments to induce cell death with camptothecin showed that in the presence of CO, the sensitivity of cells to camptothecin increased synergistically to approximately 1,000-fold over chemotherapy alone–treated cells (Fig. 2E and Supplementary Fig. S1B and S1C). The effect was specific to DNA damage–inducing agents as apoptotic responses induced by TNF and cycloheximide were not altered after CO treatment (data not shown). Further evidence of the effects of CO on PC3 cells in the presence of camptothecin showed that compared with air controls, CO induced significantly stronger cleavage of caspase-3, augmented DNA damage as measured by comet assay, and induced Gadd45α expression, an important regulator of apoptosis and DNA repair that is induced in response to chemotherapy (Supplementary Figs. S1D–S1F and S2).

Many reports detail the protective effects afforded by CO and HO-1 in response to oxidative stress in primary cells (34–36), which is in contrast to the above effects in prostate cancer cells. Treatment of normal prostate epithelial cells (PNT1A) with doxorubicin showed that unlike prostate cancer cells, CO prevented doxorubicin-induced death of normal prostate cells (Fig. 2F and Supplementary Fig. S1G) supporting the remarkable selectivity of HO-1 and CO to enhance killing of tumor cells while protecting primary cells. We confirmed these findings using primary mouse embryonic fibroblasts (MEF) treated with camptothecin or doxorubicin at the highest doses used in cancer cells and evaluated the effects of CO. As expected, we observed 75% to 90% cell death in response to both chemotherapeutics in air-treated cells. Similar to PNT1A, CO protected primary MEFs against doxorubicin-induced cell death versus air-treated cells (Supplementary Fig. S1H). The protection of primary cells afforded by CO is independent of p53, a well-characterized modulator of cell death in response to DNA damage. CO was also able to protect p53−/− MEF (Supplementary Fig. S1H).

**CO inhibits growth of human prostate cancer xenografts**

We next evaluated the role of CO alone and CO in combination with doxorubicin on the growth of tumors in vivo, using PC3 human prostate cancer xenografts implanted subcutaneously in nude mice. Mice with established tumors were exposed to a regimen of CO (250 ppm for 1 hour once a day at the same time of day) ± doxorubicin (doxorubicin was injected immediately after CO treatment) and growth was monitored over 2 weeks, at which point volumes of tumors were measured and harvested for histologic analyses. Mice treated with CO or doxorubicin alone showed significant tumor growth arrest versus controls as measured by tumor volume and Ki-67 expression and had no effect on body weight (Fig. 3A and Supplementary Fig. S3A and S3B). Combination treatment with doxorubicin plus CO showed further additive effects with regard to tumor size, proliferative index, and neovascularization (Fig. 3B–F). We did, however, observe a significant effect of CO alone or in combination with doxorubicin on cell death that was not observed with doxorubicin alone, suggesting a distinct cellular target for CO (Fig. 3G and H). Mitotic catastrophe as assessed by occurrence of aberrant nuclei after cell division was present among the p-Histone H3–positive cells in tumors treated with CO or doxorubicin (insets in Fig. 3E). Validation of the effects of CO with immunostaining showed that CO inhibited the cell-cycle marker cyclin A2 (densitometric analyses: air + doxorubicin: 1.15 ± 0.28, CO + doxorubicin: 0.68 ± 0.09; \( P < 0.05 \); Supplementary Fig. S4A and S4B). The number of Thy-1-positive fibroblasts was markedly decreased in animals receiving CO versus air treatment. It was not determined whether this was due to less infiltration or increased cell death (Supplementary Fig. S4C). Depletion of Thy-1–positive stromal cells may be an additional early molecular target of CO leading to...
accelerated cell death and inhibition of angiogenesis. The contribution of endothelial cell proliferation and recruitment of stroma cells in addition to tumor cell growth needs to be further evaluated.

**CO induces apoptosis and blocks mitosis and angiogenesis in a model of orthotopic prostate cancer in mice**

In a more clinically relevant model of orthotopic PC3 prostate cancer tumors, CO suppressed the mitotic index as measured by p-Histone H3 and CD31 staining in established tumors (8% and 6%, respectively; Fig. 4A–C). Importantly, CO significantly suppressed a key marker of invasion and early metastasis known as uPA (Fig. 4D). Furthermore, CO showed the trend toward decreased expression of the tumor markers MMP2 and VEGF; however, it did not reach significance (Fig. 4D). Similar to the subcutaneous tumor xenograft model, CO induced greater cell death in orthotopic tumors as compared with tumors from air-treated mice (Fig. 4E and F; 35% in CO versus 15% in air), suggesting that in addition to arresting growth, CO reduced the invasive and aggressive phenotype with a signature of mitochondrial collapse and tissue necrosis. This was different than that observed *in vitro* where CO alone did not affect cancer cell death yet inhibited cell cycle. In both tumor models, we observed a significant increase in the amount of CO present within the tumor tissue as well as in the liver and lung of CO-exposed mice (Fig. 4G and data not shown).

Figure 2. Nuclear HO-1 is enzymatically inactive. A and B, immunoblot of HO-1 in the nuclear (A) and cytoplasmic (B) fractions of various normal (NIH3T3 fibroblasts, NIH; primary human bronchial epithelial cells [E]; primary lung fibroblasts [F], as well as cancer cells lines (PC3-PC, A549-A5, HepG2-Hep, HeLa-He, T47D-T47, MDA-468-MD)). Data are representative of 3 independent experiments. C, HO-1 activity in the nuclear (white bars) and cytoplasmic (black bars) fractions of PC3 cells treated with camptothecin (1 μg/mL) or doxorubicin (10 μg/mL) for 4 hours. *P < 0.05. D, crystal violet staining of PC3 cells overexpressing HO-1 (black bars) or control vector (white bars) treated for 24 hours with camptothecin (1–10 μg/mL) or doxorubicin (1–10 μg/mL). #, P < 0.05, doxorubicin/camptothecin treated versus untreated; ##, P < 0.01, HO-1 versus control vector. E, dose-dependent treatment of PC3 cells with camptothecin (0.001–10 μg/mL) ± CO for 24 hours. **, P < 0.01; *, P < 0.05, CO ± camptothecin (black bars) versus air ± camptothecin (white bars). F, cell viability was measured by crystal violet staining of PNT1A normal primary prostate epithelial cells treated with doxorubicin (10 mg/mL) ± CO (black bars) or air (white bars) for 48 hours. *, CO þ doxorubicin versus air þ doxorubicin, P < 0.05.
CO prevents development of PIN/carcinoma lesions in TRAMP mice and inhibits lung carcinoma growth in Kras4bG12D transgenic mice

CO was tested in 2 additional models of spontaneously developed tumors using the prostate tumor TRAMP and the lung tumor Kras mouse models (26, 37). We started CO treatment in mice 8 to 20 weeks of age, which is after tumors are established (26, 37). Untreated mice showed a high proliferative index as measured by Ki-67 staining in the tumors showing multiple PIN foci and adenocarcinoma (Fig. 5A and B). In contrast, CO-treated mice had fewer PIN foci and significantly less Ki-67-positive cells and some of the CO-treated TRAMP mice were completely lesion-free (Fig. 5A and B). Importantly, during the progression of the disease and in contrast to the normal prostate where HO-1 is localized to the cytoplasm, HO-1 was expressed in the nucleus in the PIN lesions (Fig. 5C). Staining for the mitochondrial stress response marker mtTFA showed a significant increase in the number of mitochondria in the lesions of CO-treated TRAMP mice as compared with the neoplastic lesions in air-treated TRAMP mice at the same age (Fig. 5D).

A similar effect on tumor growth was observed in the Kras lung tumor model where we observed a significant inhibition in both the frequency (average, n = 24 in air vs. n = 15 in CO) as well as the size of the tumor nodules in the lung in animals treated with CO versus air (Fig. 5E). Proliferation of tumors was also significantly inhibited as evidenced by decreased Ki-67 staining (Fig. 5F). Longer treatment with CO resulted in further inhibition of tumor growth and size of the nodules (data not shown).

CO targets mitochondria to induce death of cancer cells

We next explored potential molecular mechanisms by which CO exerted its effects using prostate cancer cells as our model. Of note, HO-1 and CO have been shown to induce growth arrest of human cancer cells (38) as well as murine AC29 mesothelioma cells (55% less cells at day 2 and 80% at day 5 vs. air controls), which support a more global effect of CO in a different type of cancer cells. Given that CO is known to target mitochondria, and the data presented in Fig. 3, we next assessed the activity and number of mitochondria in cancer cells in the presence and absence of CO. CO increased OCR within minutes after exposure,
which was accompanied by ROS generation in PC3 cells that correlated with decreased ECAR and lactate levels (Fig. 6A–E). Use of the mitochondrial complex I poison rotenone to compare CO with another mitochondrial targeting compound and whether it would have similar effects in these cells as CO showed complete blockage of the OCR as was expected with rotenone and this effect occurred in both cancer and noncancer cells (Fig. 6B). We also tested mitochondrial potential using the JC-1 dye. As previously reported in other cell types (39), CO increased mitochondria membrane potential in PC3 cells (Supplementary Fig. S5). CO did inhibit OCR in normal cells, again suggesting a selective difference in the response of cancer cells and normal PNT1A cells to CO (Fig. 6B). The activation of mitochondrial activity by CO is likely contributes to a less cancerous phenotype. As the treatment time with CO was extended, PC3 cells showed clear G1 arrest (Fig. 6F), which corresponded to significant inhibition of metabolic activity of cancer cells as measured by SPECT (Fig. 6G). Furthermore, exposure to CO induced mitochondrial stress as evidenced by increased mtTFA, cytochrome c-positive staining, and an inhibition in the oxidized state of mitochondria (Fig. 6H–K). Metabolic screening showed that exposure of PC3 cells to CO for 6 hours resulted in a decrease in glucose metabolism and influenced synthetic pathways involved in nucleotide and amino acid synthesis, all of which corroborate the effects of CO on cancer cell growth (Table 1; Supplementary Table S1 and Supplementary Fig. S6).
Figure 5. CO arrests growth of established prostate and lung cancer. A and B, immunohistochemistry analysis with antibody against Ki-67 and histological analysis (H&E) was conducted on the tissues from wild-type (wt) or TRAMP mice that were treated with CO (250 ppm/daily/5 d/wk) or untreated (air). Quantitation of Ki-67 staining is shown in A (n = 6–7 mice per group). C, immunohistochemistry with antibodies to HO-1 was applied in the normal (wt) and PIN lesion containing prostates (TRAMP) as well as in the lung tumors of FVB/N-Tg(teto-Kras2)12Hev (provided by Dr. H. Varmus, National Cancer Institute, Bethesda, MD). Scale bar, 200 µm. D, immunostaining of prostates from TRAMP treated as above with antibodies to mitochondrial transcription factor A. Two representative pictures from air and CO-treated mice are shown. Scale bar, 200 µm. E and F, H&E staining in the lungs of FVB/N-Tg(teto-Kras2)12Hev mice that were treated with doxycycline for 8 weeks and continued with CO treatment for the following 5 weeks. The number of nodules in the lung cross-sections was evaluated in at least n = 5 to 6 per animal. The representative sections and immunohistochemistry with antibody against Ki-67 are shown in F. Data are representative for air (n = 8) and CO (n = 9 animals). P < 0.0002. Scale bar, 200 µm.
In efforts to further investigate the role of mitochondria in the effects observed with CO, we depleted mitochondria from cancer cells (Rho⁺ cells), which resulted in epithelial-to-mesenchymal transition as evident by the emergence of fibroblast-like-shaped cells and enhanced expression of vimentin in the Rho⁺ cells compared with control, which was unaffected by CO, further suggestive of mitochondrial targeting by CO (Fig. 6L).
then normal cells; however, CO did not amplify the effects of doxorubicin in these cells in contrast to normal cells (% of surviving colonies: A549: air + doxorubicin: 29.8% ± 1.2%; CO + doxorubicin: 13.4% ± 0.7%; A549Rho; air + doxorubicin: 17.1% ± 1%; CO + doxorubicin: 17.6% ± 0.39%). On the basis of changes in mitochondrial function described above and the specifically the increase in ROS generation, we next depleted ROS using a cocktail of pegylated catalase and superoxide dismutase. Addition of the antioxidant cocktail before treatment with CO resulted in reversal of the effects of CO on doxorubicin-induced apoptosis, further supporting ROS and the mitochondria as the cellular target for CO (Supplementary Fig. S7).

**Discussion**

In the present study, we elucidate a role for CO in modulation of prostate and lung tumor growth and survival. CO not only mimics the effects of chemotherapy alone by blocking proliferation but also amplifies tumor cell death when treated in combination with either doxorubicin or camptothecin. Furthermore, we provide evidence that CO reduces the tumor burden in xenograft and transgenic mouse models of prostate and lung cancers and effectively blocks progression of neoplasia and adenocarcinoma. We deduce that CO effectively switches the metabolic state of the cancer cell to fuel oxidative metabolism and decreases in nucleotide and amino acid synthetic pathways. Collectively, this causes cell-cycle arrest and collapse under intense mitochondrial-dependent oxidative stress. In vitro, this elicits a 1,000-fold increase in sensitivity to genotoxins fostered, in part, by a mitochondria-dependent increase in ROS generation. In vivo, the role of CO and ROS on tumor growth is less clear and likely involves other cellular mechanisms including hypoxia and perhaps a heightened immune response. Importantly, CO protects normal cells from DNA damage by cytotoxic agents, in part, by reducing oxygen consumption and eliciting a hibernation-like state that has been observed in other cell types such as T cells and smooth muscle cells with the induction of DNA repair complexes (40).

HO-1 is an accepted cytoprotective gene in most stress-related pathologies acting to reestablish homeostasis (41, 42). Others and we have shown that nuclear-localized HO-1 no longer possesses enzymatic activity and as such becomes highly associated with cancer and tumor growth (30, 43). We show that CO can mimic the effects of enzymatically active HO-1 and that exposure to CO or reinstating HO-1 activity results in enhanced sensitization and acceleration of apoptosis of cancer cells and tumors while protecting normal cells against chemotherapeutic toxicity. This is consistent with prior observations that HO-1–derived CO is important in DNA repair in normal cells (32). We posit that the subsequent decrease in endogenous CO generation when HO-1 is in the nucleus results in accelerated DNA stress and damage and thus serves as a hallmark of early carcinogenesis.

In our cohort of patients, nuclear HO-1 is increased in a subset of patients with prostate cancer with a shorter time to BCR. We did not observe a significant association of HO-1 expression with Gleason grades due to the relatively low number of cohort cases with the most advanced disease (Gleason grade 5). We would anticipate however statistically significant correlations in a larger cohort of patients as reported by Sacca and colleagues who showed a clear association between HO-1 and prostate cancer incidence. Inhibition of HO-1 pharmacologically using zinc-protoporphyrin (Zn-PP) induced apoptosis and suppressed growth of sarcomas in rats (44), but Zn-PP treatment failed to potentiate the antitumor effects of 5-fluorouracil, cisplatin, and doxorubicin in 3 different tumor models (45). HO-1 overexpression or CO exposure reduced TPA-induced invasion of breast cancer cells (13) as well as lung adenocarcinoma cells (38). The effects of HO-1 on tumor growth and progression are complex and likely depend on cancer types, the cell-cycle status, the model system, as well as the methods of pharmacologic or genetic manipulation of HO-1 activity.

In our model of human tumor xenografts in vivo, we observed strong inhibition of angiogenic markers, mitosis, and accelerated apoptosis in tumors treated with CO. Furthermore, CO sensitized cancer cells and solid tumors to apoptosis with apparent mitotic catastrophe likely influenced by cellular exhaustion as evidenced by our biochemical data (Fig. 6). The effects of CO on mitochondria have been well described and we speculate that the high metabolic requirements of cancer cells lend itself to greater effects of CO on survival given that CO is known to target mitochondrial respiratory complexes (33). This is supported by the effects of CO on mitochondrial membrane potential (46), biogenesis (36), and decreased prohibitin (data not shown), which regulate cancer cell survival and growth. PC-3 xenografts are partially resistant to doxorubicin-induced apoptosis, which delays tumor growth through

**Table 1.** Metabolic analysis was conducted using MetaboAnalyst 2.0

<table>
<thead>
<tr>
<th>Peaks (m/z/rt)</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-Adenosyl-L-homocysteine-positive</td>
<td>7.9552</td>
</tr>
<tr>
<td>S-Adenosyl-L-homocysteine-negative</td>
<td>7.8218</td>
</tr>
<tr>
<td>1-Methylnadensine</td>
<td>6.7669</td>
</tr>
<tr>
<td>NADH-nega</td>
<td>6.2849</td>
</tr>
<tr>
<td>2-Deoxyglucose-6-phosphate</td>
<td>6.0176</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>5.4215</td>
</tr>
<tr>
<td>Pyridoxamine</td>
<td>5.4073</td>
</tr>
<tr>
<td>NG-dimethyl-L-arginine</td>
<td>5.3147</td>
</tr>
<tr>
<td>Pyrophosphate</td>
<td>5.1344</td>
</tr>
<tr>
<td>Ribose phosphate</td>
<td>5.0937</td>
</tr>
<tr>
<td>NADH</td>
<td>5.0912</td>
</tr>
<tr>
<td>Thymine</td>
<td>5.0494</td>
</tr>
<tr>
<td>3-Phospho-serine</td>
<td>5.0256</td>
</tr>
</tbody>
</table>

**NOTE:** Top 13 of 290 metabolites were inhibited by CO (250 ppm, 6 hours) treatment of PC3 cells by more than 5-fold. n = 3 in duplicates. Univariate fold change analysis was conducted by MetaboAnalyst 2.0.
mitotic blockade (47). This effect was amplified by the presence of CO in vitro and in vivo. The increase in ROS by CO in cancer cells, likely augments protein and mitochondrial DNA (48). Indeed, inhibition of HO-1 activity in prostate cancer cells correlated with a reduction in protein carbonylation and ROS formation (49).

The finding that CO protects normal cells from genotoxin toxicity offers an added benefit and may permit chemosparing therapy, thus decreasing negative side effects. We have not tested the response to irradiation, however considering that CO protects normal cells against cell death via amplification of DNA repair (32), we expect that CO may have similar sparing effects on normal cells in response to irradiation while amplifying cancer cell death in response to irradiation. CO-elicited protection of normal cells suggests that normal cells tolerate alterations in metabolic demands more efficiently and are more capable of repairing damaged DNA. The effect of CO to enhance killing is not specific to prostate cancer cells, as we observed accelerated cell death of breast, lung, and other prostate cancer cell lines treated with doxorubicin and CO as well as dysregulated smooth muscle cells that lead to vascular stenosis in models of angioplasty, transplantation, and pulmonary hypertension. CO at these doses has no untoward effects on the animals and in fact also protects against doxorubicin-induced cardiomyopathy (36).

In summary, we show that HO-1 expression in cancer specimens is targeted to the nucleus in moderately differentiated tumor cells for reasons that remain to be elucidated. Collectively, CO influences cellular bioenergetics that we find differs between cancer cells and normal cells. CO accelerates oxidative metabolism and ROS generation, unlike in noncancer cells where CO inhibits respiration and protects against cell death. Insufficient respiration due to dysfunctional mitochondria drives what is known as the Warburg effect. Exposure to CO uses Warburg physiology as an advantage, compelling the cancer cell to consume more oxygen that in turn drives metabolic demand, leading to growth inhibition, cellular exhaustion, and death. These data provide the first evidence showing the potential for safe, low amounts of CO to be used as an adjuvant therapeutic option for the treatment of cancer. One might envision a specific inhalational therapy regimen being implemented such as that used here to treat patients with cancer with inhaled CO using a specific metered dosing delivery device or a CO releasing molecule (CO-RM; ref. 50).

Disclosure of Potential Conflicts of Interest
J. Belcher has a commercial research grant from Sangart Inc. and Seattle Genetics. No potential conflicts of interest were disclosed by the other authors.

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Study supervision: B. Wegiel, V. Sukhatme, L.E. Otterbein
Reference pathologist: L. Helczynski

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Carbon Monoxide Expedites Metabolic Exhaustion to Inhibit Tumor Growth

Barbara Wegiel, David Gallo, Eva Csizmadia, et al.


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