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Carbon monoxide releasing molecule-2 suppresses proliferation, migration, invasion, and promotes apoptosis in non-small cell lung cancer Calu-3 cells

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Abstract. – OBJECTIVE: Non-small cell lung cancer (NSCLC) is the most common type of lung cancer, which is the leading cause of cancer-related morbidity and mortality worldwide. The carbon monoxide-releasing molecules (CO-RMs) are transition metal carbonyls with the capacity to release carbon monoxide (CO). The aims of our study were to assess the effects and underlying mechanisms of CO-releasing molecules-2 (CORM-2) on proliferation, migration, invasion and apoptosis in NSCLC cells, and to evaluate its potential application for lung cancer.

MATERIALS AND METHODS: NSCLC cells Calu-3 were treated with CORM-2, negative control and blank control. Cell proliferation, migration and invasion were assessed by cell Counting Kit-8 (CCK-8), scratch assay and matrigel invasion chamber experiment, respectively. Apoptosis was measured by flow cytometry. Real-time PCR and Western blot were applied to examine the expression of apoptosis-related molecules on mRNA and protein levels.

RESULTS: CORM-2 markedly attenuated proliferation, migration and invasion of Calu-3 cells. CORM-2 treatment also significantly reduced the ratio of B cell lymphoma 2 (Bcl-2)/B cell lymphoma 2 associated X protein (Bax) while increased expression of caspase-3 and cytochrome c. The optimal dose of CORM-2 for Calu-3 cells was 100 μM.

CONCLUSIONS: CORM-2 modulates biological functions of NSCLC cells and may provide a novel therapeutic strategy for lung cancer.

Key Words
Carbon monoxide, CORM-2, NSCLC, Calu-3, Proliferation, Invasion, Apoptosis.

List of Abbreviations

NSCLC, non-small cell lung cancer; CO, carbon monoxide; CO-RMs, carbon monoxide-releasing molecules; EGFR, epidermal growth factor receptor; HO, Heme oxygenase; DMSO, dimethyl sulfoxide; CCK-8, cell counting Kit-8; OD, optical density; PI, propidium iodide; LPS, lipopolysaccharide; TPA, 12-O-tetradecanoylphorbol-13-acetate; MMP, matrix metalloproteinase.

Introduction

Despite a significant revolution in understanding the molecular pathogenesis of lung cancer in the past several decades, lung cancer remains the leading cause of cancer-related morbidity and mortality worldwide[1,2]. Non-small cell lung cancer (NSCLC) accounts for 85% of lung cancer cases[3]. The inadequacy of standard chemotherapy in advanced NSCLC has driven the development of targeted therapeutic approaches such as those directed against the epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor, and anaplastic lymphoma kinase. However, the frequency of these targets varies greatly in different populations. For example, the frequency of EGFR mutation accounts for 30-50% of lung adenocarcinoma patients in East Asia and Latin American, whereas it only occurs in 10-15% in Caucasian population[4,5]. Therefore, novel treatment options effective for all cases are urgently needed.

Heme oxygenase (HO) catalyzes the rate-limiting step in the heme degradation processes, which generate three by-products, biliverdin, iron ions and carbon monoxide (CO)[6]. HO-2 is a constitutive isoform that is expressed under homeostatic conditions, while the expression of the inducible isoform HO-1 is strictly regulated in response to a wide variety of endogenous and exogenous stimuli[7]. Thus, small amounts of CO are continuously produced in the body, and its intracellular levels can mark-

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edly increase under stressful conditions following induction of HO-1. The discovery of nitric oxide, a similar low-molecular-weight gas, as an important endogenous signaling molecule attracts growing attention to the physiological significance of HO-derived CO. Decades of research have not only elucidated CO as a cytoprotective regulator with anti-oxidative, anti-inflammatory, anti-proliferative, and vasoactive properties, but also provided possible manipulation of HO-1/CO system for therapeutic benefit in organ injury and disease[8].

CO-releasing molecules (CO-RMs) are a group of compounds, which carry and liberate controlled quantities of CO in cellular systems, therefore conquer the well-known adverse effects of CO intoxication[9,10]. CO-RMs either contain a heavy metal such as manganese (CORM-1), ruthenium (CORM-2 and -3) or iron (CORM-F3) or are based on boron (CORM-A1). New generation CO-RMs based on iron carbonyl structures, light-activated or photo-activated CO-RMs have been described[9,11]. Once in contact with myoglobin or other heme-dependent proteins that trigger the dissociation of CO from the metal, CORM-2 spontaneously releases CO. Information on adverse effect of CORM-2 is sparse.

In this study, we have investigated whether exogenous CO modulates the biological behaviors of lung cancer cells. Using NSCLC cell line Calu-2, we demonstrated that treatment with 100 μM CORM-2 has dramatically decreased the proliferation, migration and invasion of Calu-2 cells while increased their apoptosis. Furthermore, we identified that downregulation of Bcl-2/Bax ratio and upregulation of caspase-3 and cyto-c protein levels might be the underlying mechanism of increased apoptosis of Calu-2 cells treated with CORM-2.

Materials and Methods

Calu-3 Cell Culture and Treatment

The human NSCLC Calu-3 cells were grown in MEM/NEAA supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (Gibco, Grand Island, NY, USA) in a 5% CO_2 humidified incubator at 37°C. Exponential-phase cells were divided into five groups. Cells without any treatment are referred as blank control group (BC). Cells treated with 0.025% dimethyl sulfoxide (DMSO) are referred as negative control group (NC). Three doses of CORM-2 dissolved in 0.025% DMSO, 50 μM, 100 μM and 200 μM respectively, were applied in CORM-2 treatment groups for 24 h.

CCK-8 Assay

Cell Counting Kit-8 (CCK-8) was used to detect cell proliferation. Briefly, 1×10^5/ml exponential-phase cells were digested with trypsin (Invitrogen, Carlsbad, CA, USA) and seeded in 96-well tissue culture plates at a density of 1×10^4 cells per well. All groups were performed in quadruplicates. At 48, 72 and 96 h after incubation respectively, 10 μl of CCK-8 solution (Olympus, Tokyo, Japan) were added to each well, and plates were incubated at 37°C for another 1-4 h. The optical density (OD) was measured at 450 nm using a scanning multi-well spectrophotometer.

Cell Migration

Cell migration was measured using modified *in vitro* scratch assay as previously described[12]. Exponentially growing Calu-3 cells were trypsinized and resuspended to the density of 5×10^4/ml. Cells were then seeded onto 6-well cell culture plates. Once cells reached 80% confluence, scratch injury was applied using a disposable 10 μl pipette tip. After injury, cells were gently washed with DPBS, and incubated with fresh medium containing no drug (BC group), 0.025% DMSO (NC group), 50 μM, 100 μM and 200 μM CORM-2 (CORM-2 treatment groups). The injured monolayer was examined and photographed at 0 h and 24 h after scratching and migration distances were measured using Image-ProPlus 6.0 software.

Cell Invasion

Cell invasion experiments were performed using Corning™ BioCoat™ Matrigel™ Invasion Chamber (Corning, Corning, NY, USA). 200 μL Calu-3 cell suspension at the concentration of 25×10^4/ml was added into the upper compartment of the chamber while 600 μl of 10% fetal bovine serum (FBS)-Dulbecco's Modified Eagle Medium (DMEM) culture media was added to the lower chamber. After 24 h incubation at 37°C in a 5% CO_2 incubator, non-invading cells were removed by a cotton swab. The invading cells on the under surface of the filter were fixed in 4% paraformaldehyde for 15 min and stained with 0.1% Crystal Violet for 15 min. Cells invasion values were determined by counting stained cells using a light microscope (×100). Each determination represents the average of five individual fields and error bars represent standard deviation. Experiment was repeated three times.

Apoptosis Assay

Cell apoptosis was assessed using an Annexin V- fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (Sigma-Aldrich, St. Louis, MO, USA) as described previously[13]. Calu-3 cells in each group were washed and resuspended at a concentration of 1×10^5 cells/ml in 195 µl of Annexin V-FITC binding solution and 5 µl of annexin V-FITC conjugate were added. After 10 min incubation at room temperature in the dark, cells were centrifuged and resuspended in 190 µl of Annexin V-FITC binding solution followed by 10 µl of Propidium Iodide (PI) solution. Mixture was placed on ice in the dark and the fluorescence of the cells was determined within 1h with a flow cytometer.

RNA Extraction and RT-qPCR

Total RNA was extracted by TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. The quality of extracted RNAs was examined by measuring the absorbance at 260/280 nm on spectrophotometer. Quantification of Bcl-2 and Bax mRNA was performed using SYBR Green PCR kit (Qiagen, Valencia, CA, USA) with 7500 Fast Real-time PCR system (Applied Biosystems, Foster City, CA, USA) and β-actin was used as a housekeeping gene. The primers for Bcl-2 are (F) 5′- CCGCTCGAGGATCAGACCTTTGAATGATTC-3′ and (R) 5′- ATAAGAATGCGGCCGCCTCTGTGAATCCCGTTTGAA-3′. The primers for Bax are (F) 5′- TCATCCAGGATCGAGCAGAGA-3′ and (R) 5′-CCAATTCGCCGGAGACACT-3′. The primers for β-actin are (F) 5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′ and (R) 5′-TGGCACCCAGCACAATGAA-3′. Experiment was repeated three times.

Western Blot

40 mg of protein were subjected to electrophoresis with a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad, Hercules, CA, USA) and then transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were incubated with antibodies against Bcl-2, Bax, caspase-3, cyto-c and β-actin (Cell Signaling, Danvers, MA, USA) at a 1/1000 dilution overnight at 4°C after the addition of blocking buffer. Secondary antibody to IgG conjugated to horseradish peroxidase (HRP) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:5000 dilution was used. The blots were then incubated with the electrochemiluminescence (ECL) Western blot detection system according to the manufacturer's instructions. Data presented are representative of three separate experiments.

Statistical Analysis

Data were expressed as the mean value± standard deviation (mean±SD). One-way ANOVA followed by the least square difference (LSD) were performed for comparisons by using statistical software SPSS18.0 (SPSS Inc. Chicago, IL, USA). Differences were considered statistically significant at $p<0.05$.

Results

CORM-2 Inhibits Proliferation of NSCLC Cells

Previous studies revealed that overexpression of HO-1 resulted in attenuated proliferation of NSCLC cells[14,15]. Hence, to explore HO-1/CO as a potential therapeutic modality, we determined whether CORM-2 was able to decrease proliferation of Calu-3 cells. In this study, 50 µM, 100 µM and 200 µM of CORM-2 were applied to Calu-3 cells. Cell proliferation was measured at 24 h, 48 h, 72 h and 96 h after treatment using CCK-8 in all three CORM-2 treatment groups in addition to BC and NC groups. As shown in Figure 1,

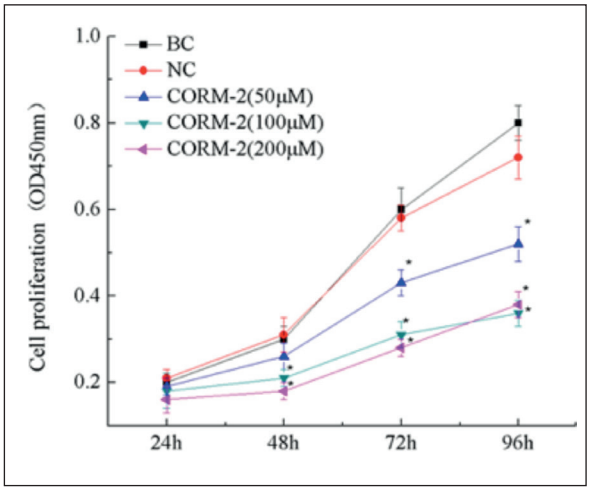

Figure 1. Attenuated cellular proliferation of Calu-3 cells treated with CORM-2. The number of proliferating cells was measured by using Cell Counting Kit-8 for 96 H. Cell proliferation (%) was calculated as OD_{450nm}. As shown in the growth curves, CORM-2 treated cells grew significantly slower than control cells. n=4, *$p<0.05$ compared with BC group, #$p<0.05$ compared with NC group.

no difference of proliferation rates was observed at 24 h among the five groups ($p > 0.05$). Calu-3 cells treated with 50 μM of CORM-2 showed significantly lower proliferation rates at 72 h and 96 h compared with that in the BC and NC groups (*$p < 0.05$ compared with BC group, #$p < 0.05$ compared with NC group). As the dose increased, the inhibitory effect of CORM-2 became more potent. As early as 48 h after treatment with 100 μM and 200 μM of CORM-2, cell proliferation was already significantly reduced (*$p < 0.05$ compared with BC group, #$p < 0.05$ compared with NC group). No difference was observed between 100 μM and 200 μM groups, indicating the 100 μM is the optimum dose of CORM.

CORM-2 Attenuates Migration and Invasion of Calu-3 NSCLC Cells

To determine the effect of CO on lung cancer cell motility, we studied the migration of Calu-3 NSCLC cells using a wound-healing assay. Calu-3 cells treated with CORM-2 were not able to close the wound within 24 h (Figure 2A lower panels). In contrast, in both BC and NC control cells, 100% of the wound were closed within 24 h (Figure 2A lower panels). The migration of Calu-3 cells treated with 50 μM of CORM-2 was significantly lower than that of cells in BC or NC group (Figure 2B, *$p < 0.05$ compared with BC group, #$p < 0.05$ compared with NC group). Higher doses of CORM-2 further decreased the migration but no difference

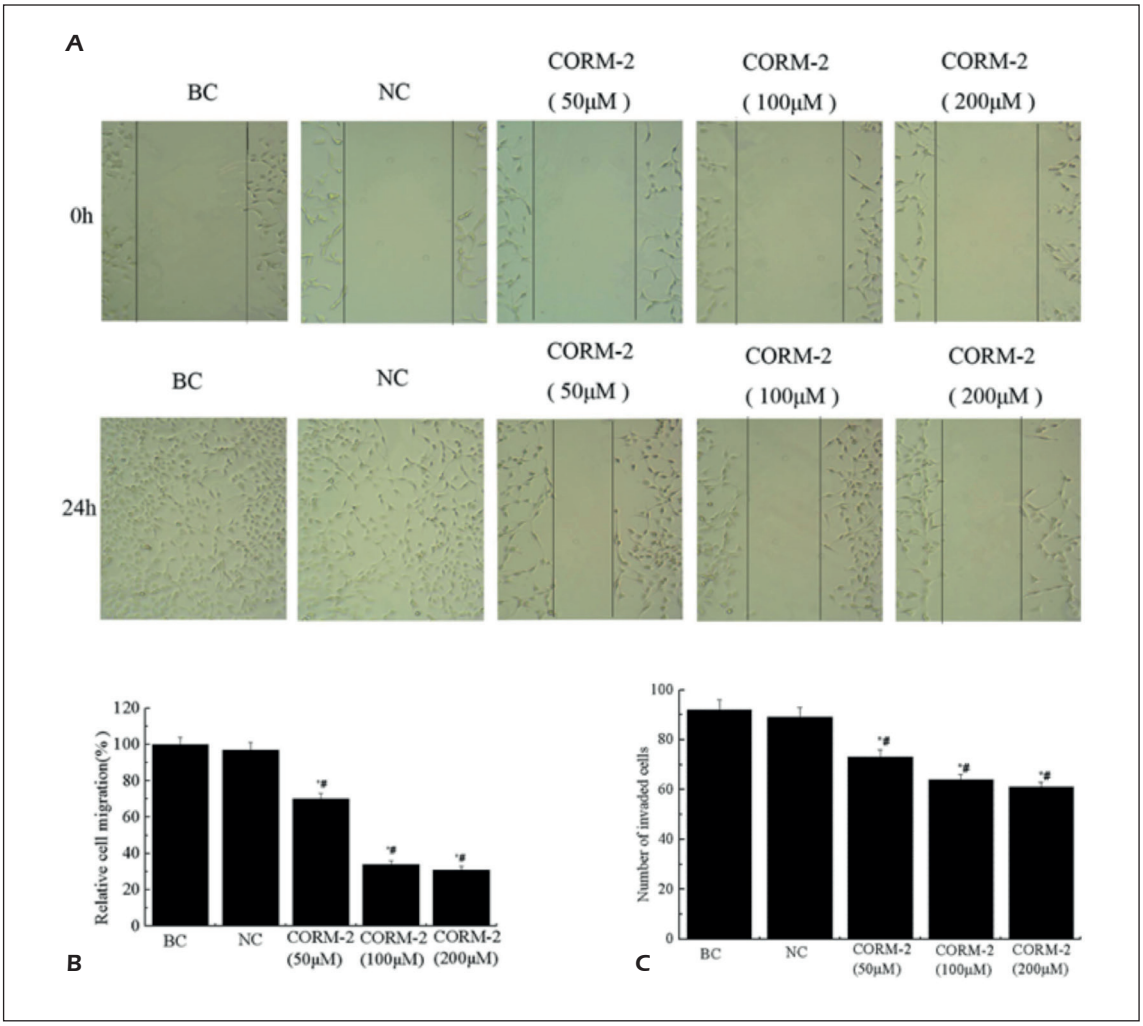

Figure 2. CORM-2 suppresses migration and invasion of Calu-3 NSCLC cells. **A**, The effect of CORM-2 on cell migration of Calu-3 NSCLC cells was tested by using scratch assay. **B**, Quantification of relative migrating distances of cells with indicated treatment. n=3, *$p<0.05$ compared with BC group, #$p<0.05$ compared with NC group. **C**, The effect of CORM-2 on cell invasion of Calu-3 NSCLC cells was tested by using transwell chamber system with Matrigel. n=3, *$p<0.05$ compared with BC group, #$p<0.05$ compared with NC group.

was observed between 100 μM group and 200 μM group (Figure 2B, $*p < 0.05$ compared with BC group, $^{\#}p < 0.05$ compared with NC group).

To examine the effect of CORM-2 on the invasive potential of NSCLC cells, we loaded the Calu-3 cells on Matrigel in the upper chamber of Transwell chambers. After incubation for 24 h, the cells that had invaded in the lower chamber were counted. Negative control treated cells had no change in invasion compared to blank control group while the invasion capacity was significantly suppressed by miR-21 inhibitor (Figure 2C, $*p < 0.05$ compared with BC group, $^{\#}p < 0.05$ compared with NC group).

Effect of CORM-2 on Apoptosis of Calu-3 Cells

Effects of HO-1 or CO on the apoptosis of cancers cells induced are controversial[16-20]. We evaluated the apoptosis of Calu-3 cells in the presence of 50 μM, 100 μM or 200 μM CORM-2 by Annexin V/PI assay. The combination of Annexin V-FITC and PI allowed us to differentiate between early apoptotic cells (Annexin V-FITC positive), late apoptotic cells (Annexin V-FITC and PI positive) and viable cells (both negative)[21]. As shown in Figure 3, CORM-2 significantly promoted early apoptotic and late apoptotic cells in a dose-dependent manner when CORM-2 was

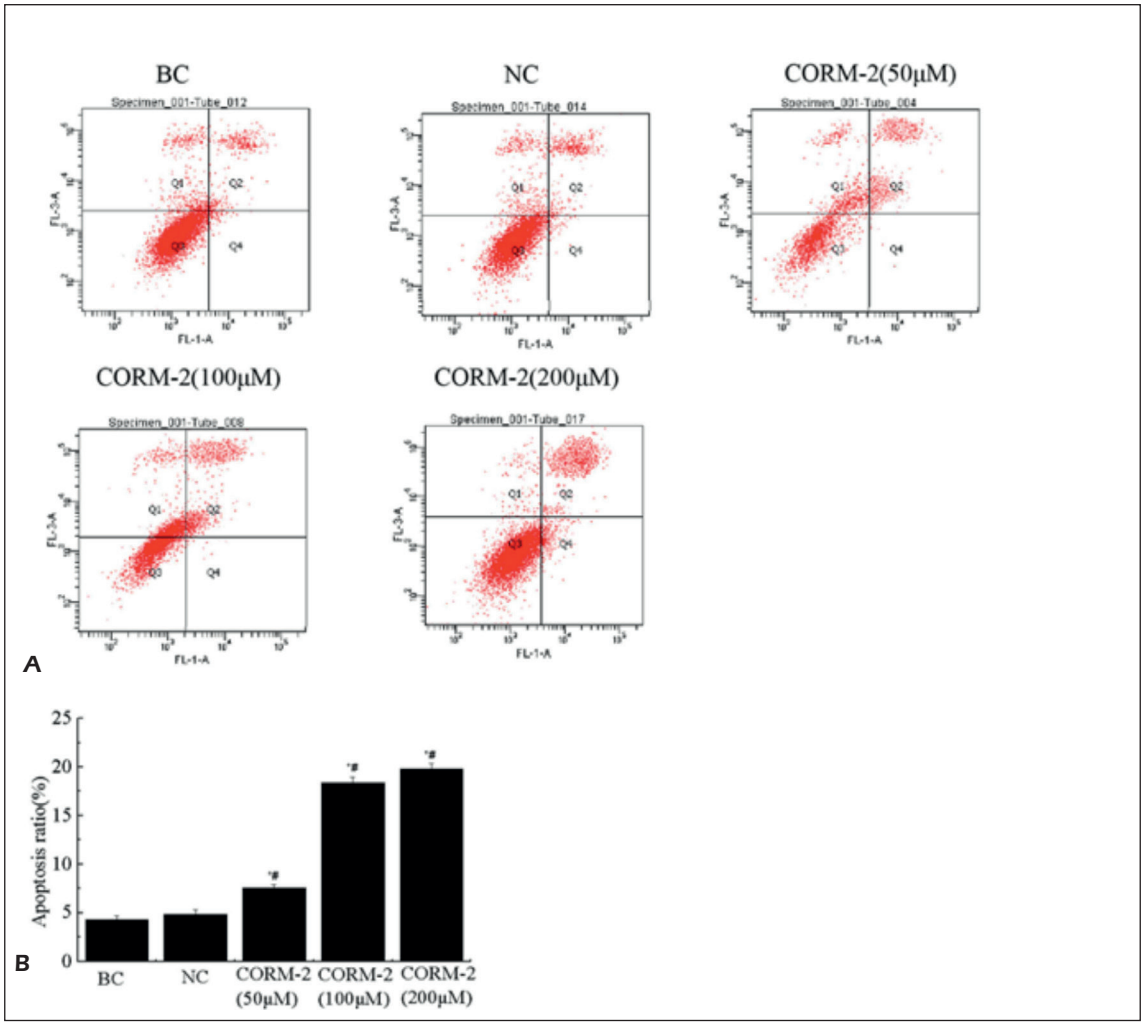

Figure 3. CORM-2 induces apoptosis in Calu-3 cells. Apoptotic cells in BC, NC and CORM-2 treated groups were measured by flow cytometry using Annexin V and propidium iodide (PI) staining. Cells in the lower left quadrant (Annexin V-FITC−/PI−) were viable cells. Those in the upper left quadrant (Annexin V-FITC+/PI−) were early apoptotic cells and those in the upper right quadrants (Annexin V-FITC+/PI+) are late apoptotic or necrotic cells. **A**, Representative scatter plots from each group. **B**, Quantification of apoptotic cells. Flow data obtained from three separate experiments were expressed as mean ± standard deviation (SD). n=3, $*p<0.05$ compared with BC group, $^{\#}p<0.05$ compared with NC group.

no more than 100 μM (Figure 3 A and B, *p< 0.05 compared with BC group, #p< 0.05 compared with NC group). As the dose of CORM-2 increased to 200 μM, apoptosis of Calu-3 cells did not increase proportionally, indicating the optimum dose might be around 100 μM.

Apoptosis Related Genes Are Regulated by CORM-2

The ratio of death antagonists Bcl-2 to agonists Bax determines whether a cell will respond to an apoptotic signal. To further identify the molecular basis underlying increased apoptosis by CORM-2 treatment, we examined expression levels of apoptosis related genes by qPCR and found that Bcl-2 mRNA was downregulated in the CORM-2 treated groups (Figure 4A, *p < 0.05 compared with BC group, #p < 0.05 compared with NC group). In contrast, the pro-apoptotic molecule Bax expression was upregulated with the same treatment compared with negative control and blank control groups (Figure 4B, *p < 0.05 compared with BC group, #p < 0.05 compared with NC group). Similar to what we found in apoptosis assay, 50 μM and 100 μM of CORM-2 altered the Bcl-2 and Bax mRNA in a dose dependent way. CORM-2 at 200 μM also significantly changed the expression of Bcl-2 and Bax mRNA compared with BC or NC groups but no difference was observed when compared with 100 μM CORM-2 group (Figure 4).

CORM-2 Regulates Apoptosis Related Proteins

One of the most prominent molecular markers of apoptosis is the massive activation of the cysteine-aspartic acid protease (caspase) family. Caspase-3 is crucial for programmed cell death and its polymorphisms define an individual's genetic susceptibility to cancer development[22, 23]. Cytochrome c (cyto-c) is a small heme protein that functions as a central component of the electron transport chain in mitochondria and the release of mitochondrial cyto-c into the cytoplasm is commonly used as an indicator of the apoptotic process in the cell[24]. We assessed whether the exogenous CO affected the protein expression of Bcl-2, Bax, caspase-3 and cyto-c by Western blot in the human NSCLC Calu-3 cells which were treated with 50-200 μM of CORM-2, negative control or blank control. CORM-2 significantly decreased Bcl-2 protein level while increased Bax production (Figure 5A), which was consistent with the changes of Bcl-2 and Bax mRNA level (Figure 4). Accordingly, the ratio of Bcl-2/Bax was significantly lower in CORM-2 treated groups compared with blank controls or blank controls (Figure 5B, *p< 0.05 compared with BC group, #p< 0.05 compared with NC group). The same treatment profoundly upregulated the caspase-3 and cyto-c levels (Figure 5A, C and D, *p< 0.05 compared with BC group, #p< 0.05 compared with NC group). Similar to the effect of CORM-2 on gene expression of apoptosis related molecules, 50 μM and 100 μM of CORM-2 altered the protein levels of Bcl-2, Bax, caspase-3 and cyto-c in a dose dependent way. As the dose increased to 200 μM, the protein levels of these apoptosis related molecules didn't change proportionally, indicating the optimum dose might be around 100 μM (Figure 5).

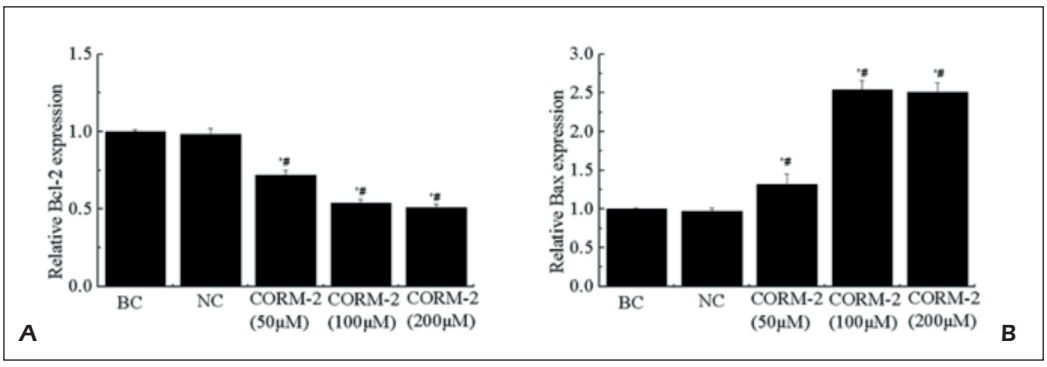

Figure 4. Apoptosis related genes are regulated by CORM-2. Expression level of apoptosis related genes was carried out by qPCR. **A**, Bcl-2 mRNA was downregulated in the CORM-2 treated groups. n=3, *p<0.05 compared with BC group, #p<0.05 compared with NC group. **B**, The pro-apoptotic molecule Bax expression was upregulated with the same treatment compared to negative control and blank control groups. n=3, *p<0.05 compared with BC group, #p<0.05 compared with NC group.

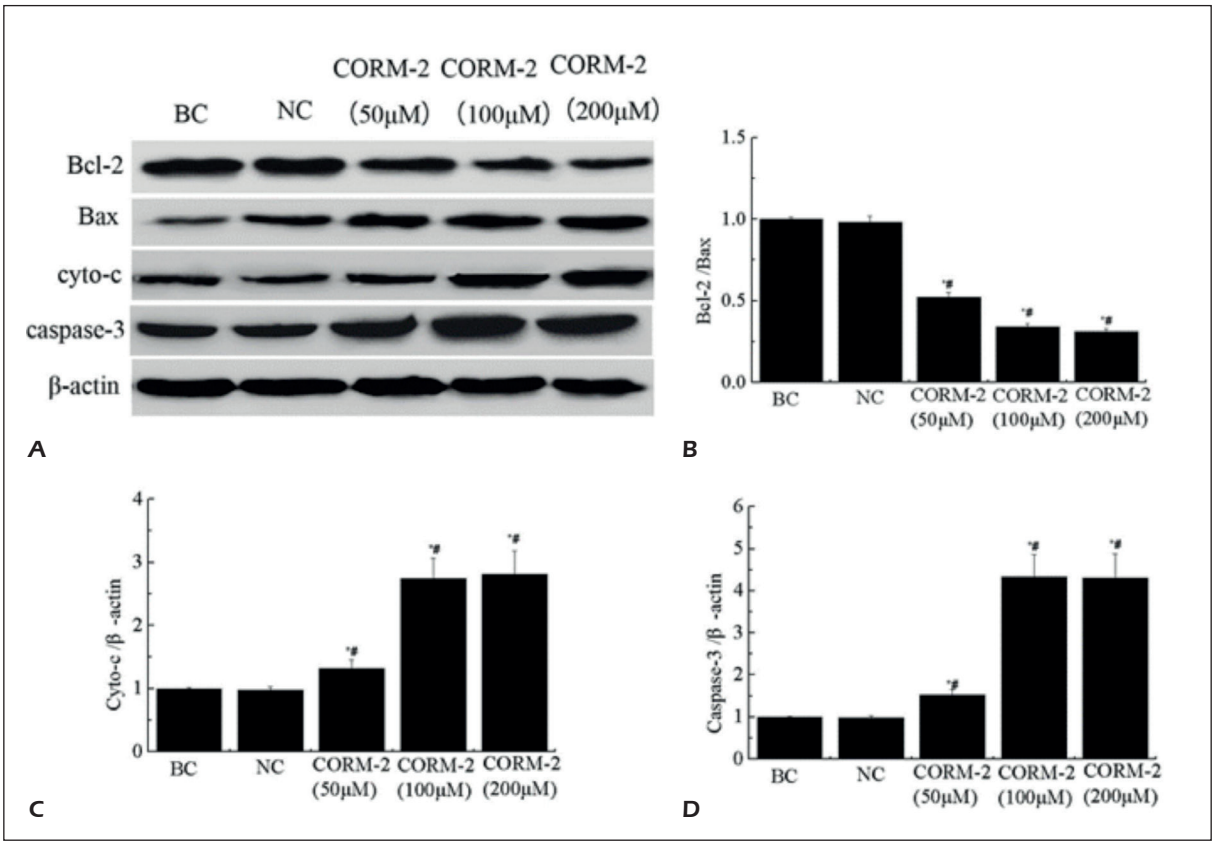

Figure 5. Effect of CORM-2 on the protein levels of apoptosis related molecules. **A**, Representative Western blot from three independent experiments. **B-D**, Densitometry of Western blot to show the ratio of Bcl-2/Bax (**B**), cyto-c/β-actin (**C**) and caspase-3/β-actin (**D**). n=3, *p<0.05 compared with BC group, $^{\#}p$<0.05 compared with NC group.

Discussion

Carbon monoxide, a colorless and odorless gas, was found to be produced endogenously in man through the oxidative breakdown of heme by microsomal heme oxygenases[25]. In addition, to activate soluble guanylate cyclase/cyclic guanosine monophosphate system and act as an intracellular messenger molecule, a variety of pathological conditions, including inflammation, cancer, hematological diseases, cardiovascular diseases, sepsis and neurodegenerative diseases, have been linked to abnormal endogenous CO metabolism and functions[26-29].

Otterbein group applied low concentration CO to cultured macrophages challenged with lipopolysaccharide (LPS) and demonstrated that LPS-dependent production of proinflammatory cytokines (i.e., TNF-α, IL-1β, and MIP-1β) was inhibited by CO whereas the anti-inflammatory cytokine IL-10 was increased[30]. The anti-proliferative effect of CO was first reported by Morita et al[31] in cultured vascular smooth muscle cells. The authors found that increasing CO production or exposing cells to exogenous CO lead to a marked attenuation of growth response whereas inhibiting CO formation or scavenging CO with hemoglobin increased vascular smooth muscle cell proliferation in response to mitogens. Similar anti-proliferative effect of CO was also observed in lymphocytes[32]. Our work showed that exposing NSCLC Calu-3 cells to CORM-2, the pharmacological application of CO, also lead to a markedly attenuated cell proliferation. Although we didn't study the mechanism underlying the antiproliferative action of CO, others suggested that CO inhibited cell proliferation by changing the expression and/or activation of cell cycle-related factors. In smooth muscle cells, CO treatment induced $P21^{Waf1/Cip1}$ expression, which is a cell cycle inhibitor and controls cell cycle-dependent kinases and cyclin complexes[33]. ERK1/2 downregulation and decreased cyclin D expression as a consequence of inhibited mitochondrial respiratory chain and NAD(P)H oxidase might be another mechanism[34].

In addition to the anti-proliferative activity, CO has been shown to regulate tumor cells migration and invasion. A previous study found that elevation of HO-1 gene expression inhibited 12-O-tetradecanoylphorbol-13-acetate (TPA) induced matrix metalloproteinase-9 (MMP-9) activation and subsequent migration and invasion of MCF-7 breast cancer cells. Additionally, the addition of CO, but not ferric ions, biliverdin, or bilirubin, inhibited TPA-induced invasion through suppressing MMP-9[35]. Works on pleural mesothelial cells showed similar results. Carbon monoxide or the induction of HO-1 inhibited the expression of myofibroblast markers and migration in pleural mesothelial cells treated with TGF-β1[36]. We performed scratch assay and matrigel invasion experiment to investigate the effects of CO on NSCLC cells. Calu-3 cells treated with CORM-2 had significant reduction in migration and invasion compared with untreated cells. Tumor migration and invasion are the initial steps in metastasis. Our study suggested that CORM-2 may be candidates for chemoprevention.

Growing evidence supports an important role of CO in the regulation of cell apoptosis. However, studies regarding to the exact effect of CO in apoptosis are still controversial. A previous report on human prostate cancers found that exposure to CO sensitized cancer cells but not normal cells to chemotherapy, with growth arrest and apoptosis induced *in vivo*[19]. Nemeth et al[20] demonstrated that CO treatment, especially low doses of CO, induced apoptosis in lung tumors though modulation of macrophages in the tumor microenvironment. In current study, CO treatment in the form of CORM-2 induced NSCLC cells apoptosis via down-regulation of anti-apoptotic molecule Bcl-2 and up-regulation of pro-apoptotic molecule Bax and subsequent apoptosis-related molecules caspase-3 and cyto-c. However, work on medulloblastoma DAOY cells showed that CO mediated the anti-apoptotic effects of HO-1 via K+ channel inhibition[18]. Anti-apoptotic effect of CO was also found in non-tumor neural cells[37,38]. The CORM ALF-186 mediated anti-apoptotic signaling via an activation of the p38 MAPK after ischemia and reperfusion injury in retinal ganglion cells [37]. Low-dose CO inhalation protects neuronal cells from apoptosis after optic nerve crush[38]. These results indicate that the effect of CO on cell apoptosis is complex and likely depends on cell and/or cancer types, the cell cycle status, the model system, as well as the methods of pharmacologic manipulation of CO.

In order to conquer the difficulties in handling a gaseous agent and the negative effects of inhaled CO on oxygen transport, carbon monoxide-releasing molecules (CO-RMs) capable of delivering a predictable and controllable amount of CO to organs and tissues were first developed by Motterlini et al[39]. Megias et al[40] showed that concentrations of CORM-2 up to 150 μM didn't affect the viability of colon cancer cells. Although we didn't observe any cellular toxicity in lung cancer cells treated with CORM-2 up to 200 μM, the anti-cancer effects of CORM-2 didn't increase when the dose of CORM-2 went up from 100 μM to 200 μM, indicating an optimum dose of 100 μM in our system.

In the present study, we elucidate a role for carbon monoxide in modulation of non-small cell lung cancer cells growth and survival. CORM-2 derived CO not only blocks proliferation, migration and invasion, but also promotes tumor cell death. Further, we provide evidence that CO inhibits expression of anti-apoptotic molecule Bcl-2, while induces expression of apoptosis related molecules Bax, caspase-3 and cyto-c. Collectively, this causes cell apoptosis. In NSCLC Calu-3 cells, CORM-2 achieves its maximum effect at the dose of 100 μM.

Conclusions

Overall, our study is the first to suggest that CO-RMs may possess chemoprotective properties in NSCLC and, in this particular case, may regulate cancer cell proliferation, migration, invasion and apoptosis via modulation of apoptosis related molecules. Our report opens another door for these exciting molecules in the future management of the leading health killer.

Conflict of Interest

The authors declare that there is no conflict of interest.

References

1) GLOBOCAN 2012: Estimated Cancer Incidence, Mortality and Prevalence Worldwide in 2012, URL://http://globocan.iarc.fr., (2012).
2) Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin 2015; 65: 87-108.
3) Herbst RS, Heymach JV, Lippman SM. Lung cancer. N Engl J Med 2008; 359: 1367-1380.

4) Shigematsu H, Lin L, Takahashi T, Nomura M, Suzuki M, Wistuba II, Fong KM, Lee H, Toyooka S, Shimizu N, Fujisawa T, Feng Z, Roth JA, Herz J, Minna JD, Gazdar AF. Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. J Natl Cancer Inst 2005; 97: 339-346.
5) Arrieta O, Cardona AF, Martín C, Más-López L, Corrales-Rodríguez L, Bramuglia G, Castillo-Fernandez O, Meyerson M, Amieva-Rivera E, Campos-Parra AD, Carranza H, Gómez de la Torre JC, Powazniak Y, Aldaco-Sarvide F, Vargas C, Trigo M, Magallanes-Maciel M, Otero J, Sánchez-Reyes R, Cuello M. Updated frequency of EGFR and KRAS mutations in nonsmall-cell lung cancer in Latin America: the Latin-American Consortium for the Investigation of Lung Cancer (CLICaP). J Thorac Oncol 2015; 10: 838-843.
6) Tenhunen R, Marver HS, Schmid R. Microsomal heme oxygenase. Characterization of the enzyme. J Biol Chem 1969; 244: 6388-6394.
7. Bauer M, Huse K, Settmacher U, Claus RA. The heme oxygenase-carbon monoxide system: regulation and role in stress response and organ failure. Intensive Care Med 2008; 34: 640-648.
8. Ryter SW, Choi AM. Heme oxygenase-1/carbon monoxide: from metabolism to molecular therapy. Am J Respir Cell Mol Biol 2009; 41: 251-260.
9. Motterlini R. Carbon monoxide-releasing molecules (CO-RMs): vasodilatory, anti-ischaemic and anti-inflammatory activities. Biochem Soc Trans 2007; 35: 1142-1146.
10. Chatterjee PK. Water-soluble carbon monoxide-releasing molecules: helping to elucidate the vascular activity of the 'silent killer'. Br J Pharmacol 2004; 142: 391-393.
11. Ryter SW, Choi AM. Targeting heme oxygenase-1 and carbon monoxide for therapeutic modulation of inflammation. Transl Res 2016; 167: 7-34.
12. Liang CC, Park AY, Guan JL. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. Nat Protoc 2007; 2: 329-333.
13. Yang J, Zhang HF, Qin CF. MicroRNA-217 functions as a prognosis predictor and inhibits pancreatic cancer cell proliferation and invasion via targeting E2F3. Eur Rev Med Pharmacol Sci 2017; 21: 4050-4057.
14. Skrzypek K, Tertil M, Golda S, Ciesla M, Weglarczyk K, Collet G, Guichard A, Kozakowska M, Boczkowski J, Was H, Gil T, Kuzdzal J, Muchova L, Vitek L, Loboda A, Jozkowicz A, Kieda C, Dulak J. Interplay between heme oxygenase-1 and miR-378 affects non-small cell lung carcinoma growth, vascularization, and metastasis. Antioxid Redox Signal 2013; 19: 644-660.
15. Tertil M, Skrzypek K, Florczyk U, Weglarczyk K, Was H, Collet G, Guichard A, Gil T, Kuzdzal J, Jozkowicz A, Kieda C, Pichon C, Dulak J. Regulation and novel action of thymidine phosphorylase in non-small cell lung cancer: crosstalk with Nrf2 and HO-1. PLoS One 2014; 9: e97070.
16. Liu ZM, Chen GG, Ng EK, Leung WK, Sung JJ, Chung SC. Upregulation of heme oxygenase-1 and p21 confers resistance to apoptosis in human gastric cancer cells. Oncogene 2004; 23: 503-513.
17. Kocanova S, Buytaert E, Matroule JY, Piette J, Golab J, de Witte P, Agostinis P. Induction of heme-oxygenase 1 requires the p38MAPK and PI3K pathways and suppresses apoptotic cell death following hypericin-mediated photodynamic therapy. Apoptosis 2007; 12: 731-741.
18. Al-Owais MM, Scragg JL, Dallas ML, Boycott HE, Warburton P, Chakrabarty A, Boyle JP, Peers C. Carbon monoxide mediates the anti-apoptotic effects of heme oxygenase-1 in medulloblastoma DAOY cells via K+ channel inhibition. J Biol Chem 2012; 287: 24754-24764.
19. Wegiel B, Gallo D, Csizmadia E, Harris C, Belcher J, Vercellotti GM, Penacho N, Seth P, Sukhatme V, Ahmed A, Pandolfi PP, Helczynski L, Bjartell A, Persson JL, Otterbein LE. Carbon monoxide expedites metabolic exhaustion to inhibit tumor growth. Cancer Res 2013; 73: 7009-7021.
20. Nemeth Z, Csizmadia E, Vikstrom L, Li M, Bisht K, Feizi A, Otterbein S, Zuckerbraun B, Costa DB, Pandolfi PP, Fillinger J, Döme B, Otterbein LE, Wegiel B. Alterations of tumor microenvironment by carbon monoxide impedes lung cancer growth. Oncotarget 2016; 7: 23919-23932.
21. Salvucci O, Carsana M, Bersani I, Tragni G, Anichini A. Antiapoptotic role of endogenous nitric oxide in human melanoma cells. Cancer Res 2001; 61: 318-326.
22. Porter AG, Jänicke RU. Emerging roles of caspase-3 in apoptosis. Cell Death Differ 1999; 6: 99-104.
23. McIlwain DR, Berger T, Mak TW. Caspase functions in cell death and disease. Cold Spring Harb Perspect Biol 2013; 5: a008656.
24. Narita M, Shimizu S, Ito T, Chittenden T, Lutz RJ, Matsuda H, Tsujimoto Y. Bax interacts with the permeability transition pore to induce permeability transition and cytochrome c release in isolated mitochondria. Proc Natl Acad Sci U S A 1998; 95: 14681-14686.
25. Coburn RF, Blakemore WS, Forster RE. Endogenous carbon monoxide production in man. J Clin Invest 1963; 42: 1172-1178.
26. Wu L, Wang R. Carbon monoxide: endogenous production, physiological functions, and pharmacological applications. Pharmacol Rev 2005; 57: 585-630.
27. Bilban M, Haschemi A, Wegiel B, Chin BY, Wagner O, Otterbein LE. Heme oxygenase and carbon monoxide initiate homeostatic signaling. J Mol Med (Berl) 2008; 86: 267-279.
28. Ghosh S, Gal J, Marczin N. Carbon monoxide: endogenous mediator, potential diagnostic and therapeutic target. Ann Med 2010; 42: 1-12.
29. Owens EO. Endogenous carbon monoxide production in disease. Clin Biochem 2010; 43: 1183-1188.
30. Otterbein LE, Bach FH, Alam J, Soares M, Tao Lu H, Wysk M, Davis RJ, Flavell RA, Choi AM. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. Nat Med 2000; 6: 422-428.
31. Morita T, Mitsialis SA, Koike H, Liu Y, Kourembanas S. Carbon monoxide controls the proliferation of hypoxic vascular smooth muscle cells. J Biol Chem 1997; 272: 32804-32809.

32. Song R, Mahidhara RS, Zhou Z, Hoffman RA, Seol DW, Flavell RA, Billiar TR, Otterbein LE, Choi AM. Carbon monoxide inhibits T lymphocyte proliferation via caspase-dependent pathway. J Immunol 2004; 172: 1220-1226.
33. Otterbein LE, Zuckerbraun BS, Haga M, Liu F, Song R, Usheva A, Stachulak C, Bodyak N, Smith RN, Csizmadia E, Tyagi S, Akamatsu Y, Flavell RJ, Billiar TR, Tzeng E, Bach FH, Choi AM, Soares MP. Carbon monoxide suppresses arteriosclerotic lesions associated with chronic graft rejection and with balloon injury. Nat Med 2003; 9: 183-190.
34. Taillé C, El-Benna J, Lanone S, Boczkowski J, Motterlini R. Mitochondrial respiratory chain and NAD(P)H oxidase are targets for the antiproliferative effect of carbon monoxide in human airway smooth muscle. J Biol Chem 2005; 280: 25350-25360.
35. Lin CW, Shen SC, Hou WC, Yang LY, Chen YC. Heme oxygenase-1 inhibits breast cancer invasion via suppressing the expression of matrix metalloproteinase-9. Mol Cancer Ther 2008; 7: 1195-1206.
36. Zolak JS, Jagirdar R, Surolia R, Karki S, Oliva O, Hock T, Guroji P, Ding Q, Liu RM, Bolisetty S, Agarwal A, Thannickal VJ, Antony VB. Pleural mesothelial cell differentiation and invasion in fibrogenic lung injury. Am J Pathol 2013; 182: 1239-1247.
37. Ulbrich F, Kaufmann KB, Meske A, Lagrèze WA, Augustynik M, Buerkle H, Ramao CC, Biermann J, Goebel U. The CORM ALF-186 mediates anti-apoptotic signaling via an activation of the p38 MAPK after ischemia and reperfusion injury in retinal ganglion cells. PLoS One 2016; 11: e0165182.
38. Chen Z, Wang R, Wu J, Xia F, Sun Q, Xu J, Liu L. Low-dose carbon monoxide inhalation protects neuronal cells from apoptosis after optic nerve crush. Biochem Biophys Res Commun 2016; 469: 809-815.
39. Motterlini R, Clark JE, Foresti R, Sarathchandra P, Mann BE, Green CJ. Carbon monoxide-releasing molecules: characterization of biochemical and vascular activities. Circ Res 2002; 90: E17-24.
40. Megías J, Busserolles J, Alcaraz MJ. The carbon monoxide-releasing molecule CORM-2 inhibits the inflammatory response induced by cytokines in Caco-2 cells. Br J Pharmacol 2007; 150: 977-986.