Original Paper

Carbon Monoxide Inhibits Receptor Activator of NF- κ B (RANKL)-Induced Osteoclastogenesis

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Key Words

Carbon monoxide • c-Fos; MAPK • Osteoclast differentiation • PPAR- γ • RANKL

Abstract

Background: Low concentrations of carbon monoxide (CO) have anti-inflammatory effects and can reduce bone erosion in a murine collagen-induced arthritis model. The objective of this study was to assess the effects of CO on receptor activator of NF- κ B ligand (RANKL), one of the key stimulators of osteoclastogenesis. **Methods:** The *in vivo* effects of CO on RANKL expression were assessed in a collagen antibody-induced arthritis model in mice. Cell proliferation and apoptosis were assessed in the RAW246.7 cell line stimulated with RANKL and exposed to either air or CO. The number of tartrate resistant acid phosphatase (TRAP)-positive RAW246.7 cells was also examined after treatment with RANKL and the peroxisome proliferator-activated receptor gamma (PPAR γ) agonist, Troglitazone. **Results:** CO reduced RANKL expression in the synovium of arthritic mice. Although CO slightly increased RAW246.7 cell proliferation, no differences in activated caspase 3 levels were detected. In addition, Troglitazone ameliorated the inhibitory effects of CO on RANKL-induced TRAP expression by RAW246.7 cells. **Conclusions:** CO suppresses osteoclast differentiation by inhibiting the RANKL-induced activation of PPAR- γ . Given the role of the PPAR- γ /cFos (AP-1) pathway in regulating the transcription factor, NFATc1, the master regulator of osteoclastogenesis, further studies are warranted to explore CO in treating inflammatory bone disorders.

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Introduction

The process of bone remodeling by osteoblasts and osteoclasts is a sequential cycle that is strictly regulated [1]. An imbalance in normal bone homeostasis, including dysregulated osteoclast differentiation and function, underlies many inflammatory and metabolic bone diseases, such as rheumatic arthritis (RA), septic arthritis and periprosthetic osteolysis; therefore, elucidating the mechanisms that regulate this process may identify novel therapeutic targets for diseases in which osteoclastogenesis is altered.

Osteoclast differentiation can be regulated by immune cells, including T cells and natural killer cells that induce production of receptor activator of nuclear factor- κ B (NF- κ B) ligand (RANKL) [2], a tumor necrosis factor (TNF)-family cytokine [3] that regulates dendritic-cell and T-cell function and survival [4] and is a key driver of osteoclastogenesis and osteoclastic bone resorption [5, 6]. Moreover, RANKL is not only expressed by osteoblasts but also by activated T cells [7], and many inflammatory bone diseases causing bone loss or bone destruction are RANKL-dependent [8]. Given the importance of RANKL in inflammatory bone disease [9], factors that modulate its expression may have therapeutic value. For example, denosumab, a human anti-RANKL monoclonal antibody, is currently being used in osteoporosis and cancer-related bone disease treatment [9, 10].

The expression of RANKL can be modulated by many factors, such as inflammatory cytokines (e.g., tumor necrosis factor- α [TNF- α] and interleukin-1 β [IL-1 β]), dexamethasone, 1,25 dihydroxyvitamin D3 and parathyroid hormone. In contrast, estrogen and transforming growth factor β (TGF- β) reduce RANKL expression [7, 11]. Impaired osteoclast differentiation was observed in TRPV6^{-/-} knockout mice, possibly resulted from deficiencies in signaling pathways evoked by RANKL and M-CSF, such as NF- κ B pathway [12]. Furthermore, carbon monoxide (CO) inhibits RANKL signaling and therefore osteoclastogenesis, resulting in decreased bone loss associated with ovariectomy in mice [13], and treatment with CO releasing molecule-2 (CORM-2) inhibited RANKL-induced osteoclastogenesis in primary cultures of osteoclast precursors in a dose-dependent manner [14]. In a murine collagen-induced arthritis model, CO reduced inflammation and decreased the number of osteoclasts [15], and CORM reduced RANKL expression and joint destruction [16]. However, the mechanisms by which CO elicit these effects remain unknown.

CO, a well known ubiquitous air pollutant, is invisible and lacks odor. The binding affinity of CO for heme is 240 times as compared with oxygen; thus, it can cause hypoxia and death [17]. In addition to environmental sources, mammals produce endogenous CO as a result of heme degradation. Low concentrations of CO (250 ppm) elicit anti-apoptotic and anti-inflammatory effects in different models [18-21] by inhibiting macrophages, which are osteoclast precursors [13]. Furthermore, inhibition of heme oxygenase-1, which converts heme to CO, suppressed osteoclast differentiation [22]. In RANKL-stimulated RAW 246.7 cells, we previously showed that CO suppressed the number of tartrate resistant acid phosphatase (TRAP)-positive and actin-positive cells [23]. We also showed that CO reduced the expression markers of osteoclast differentiation [23], including cathepsin K, calcitonin receptor, acid phosphatase 5 (ACP5 ;TRAP), matrix metalloproteinase-9 (MMP9) [24], and the master regulator of osteoclastogenesis, nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) [25]. Moreover, CO suppressed c-Fos expression and c-Jun and JNK phosphorylation [23]. Therefore, the present study was undertaken to analyze the effects of CO in a murine collagen-induced arthritis model. In addition, the effects of CO on cell proliferation and apoptosis were assessed using the RAW 246.7 murine macrophage cell line that can be induced to differentiate into osteoclasts with stimulation by RANKL [26]. Finally, given the role of the nuclear receptor, PPAR- γ , in modulating osteoclastogenesis by directly regulating c-Fos expression [27], we examined the effects of CO and a PPAR- γ agonist on *in vitro* osteoclastogenesis.

Materials and Methods

Reagents and antibodies

RANKL was obtained from PeproTech (London, UK). The Leukocyte Acid Phosphatase Assay Kit was purchased from Sigma (St. Louis, MO, USA). D-minimal essential medium was obtained from Gibco (Carlsbad, CA, USA). Antibodies specific for caspase-3 and RANKL are from Abcam (Cambridge, UK); anti-actin antibodies are from Sigma.

Induction of an in vivo model of arthritis

Male BALB/cBYJNarl (BALB/c) mice 6 weeks of age were purchased from the National Laboratory Animal Center (Taiwan) and kept under specific pathogen-free conditions in the Laboratory Animal Center of the National Defense Medical Center (Taipei, Taiwan).

Anti-type II collagen antibody-induced arthritis (CAIA) in 8-week old mice was induced as previously described by Terato et al. [28] and is shown in Figure 1. Briefly, on day 0, 2 mg of an Arthrogen-CIA® monoclonal antibody cocktail (Chondrex, Redmond, WA, USA) in 200 µL was injected intravenously. At day 3, the mice received an intraperitoneal injection of 50 µg lipopolysaccharide (LPS) from *Escherichia coli* 111b4 in 100 µL. Animals were then sacrificed on the tenth day. The synovium of the knee was collected for further analysis by resection of the quadriceps followed by scraping of the synovial membrane adjacent to the tibia using a scalpel. All animal experiments were carried out with the approval of Institutional Animal Care and Use Committee at the National Defense Medical Center.

CO exposure

Animals were exposed to air, or air plus CO (250 ppm) continuously 7 days before arthritis induction to 10 days after antibody injection. Cells were exposed to the ordinary incubation conditions (37°C, 5% CO₂) with or without CO. CO was supplied at a concentration of 1% (10,000 ppm) in compressed air and then mixed with fresh air by an air mixer to adjust the concentration to 250 ppm before delivery into the incubator. A CO analyzer with a sensitivity of 10–600 ppm was used to measure CO levels.

H₂O₂ exposure

To test the toxicity of low concentration of CO, cell proliferation assay was performed. H₂O₂ (100µM) treated cells were used as positive control in apoptosis.

In vitro osteoclast differentiation

The murine macrophage cell line, RAW 246.7, obtained from the Bioresource Collection and Research Center (Taiwan) was cultured in 10-cm dishes to confluency then subcultured to 24-well dishes (1×10⁴ cells per well) with additional sterile glass coverslips in each well. Cells were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin solution and maintained in 37°C humidified incubator with 5% CO₂ or plus CO. RANKL (20 ng/mL) was added with and without 0.5µM Troglitazone. The medium was replaced every 2-3 days. After 5 days, the cells were fixed for TRAP staining following the manufacturer's instructions (Sigma). Osteoclasts are identified under a light microscope as TRAP-positive cells with more than three nuclei. The proportion of TRAP-positive cells was determined from 50 fields (50 images were taken for each field) under 200x magnification. This experiment was repeated three times, and the cells were counted independently by three researchers.

Real-time PCR analysis

Total RNA from the synovial membrane extracts was isolated with Trizol. For cDNA synthesis, 5 µg of total RNA was reverse transcribed at 42°C for 60 min using a RevertAid First Strand cDNA Synthesis kit set (Fermentas, Pittsburgh, PA, USA), and the reaction was terminated by heating at 75°C for 5 min. In addition to the Maxima SYBR Green/ROX qPCR

Fig. 1. Induction of the collagen antibody-induced arthritis model.

master mix kit (Fermentas) the following primer sequences were used for the real-time PCR reactions: RANKL sense, 5'-CCTGA GGCC AGCCA TTT-3' and antisense, 5'-CTTGG CCCAG CCTCG AT-3'; cathepsin K sense, 5'-ATGTGGGGGCTCAAGTTCTG-3' and antisense, 5'-CATATGGGAAAGCATCTTCAGAGT C-3'; TRAP sense, 5'-AGCAGCCAAGGAGGACTACGTT-3' and antisense, 5'-TCGTTGATGTCGCACAGAGG-3'; calcitonin receptor sense, 5'-AGTTGCCCTCTTATGAAGGAGAAG-3' and antisense, 5'-GGAGTGTCTGCCAGCACAT-3'; MMP-9 sense, 5'-GGAAGTCACACGACATCTCCA-3' and antisense 5'-GAAACTCACACGCCAGAAGAATT-3'; and GAPDH, 5'-TGAAGCAGGCATCTGAGGG-3' and 5'-CGAAGTGGAAGAGTGGGAG-3'. PCR reactions for each sample were done in triplicate.

Western blot analysis

Cytosolic and nuclear protein extracts were prepared from the synovial membrane extracts with a ProteoJET Cytoplasmic and Nuclear Protein Extraction kit (Fermentas). Cell lysates were resolved via 10% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Membranes were blocked for 1 h with 5% skim milk in TBST buffer at room temperature after which they were incubated for 1 h at room temperature or overnight at 4°C with primary antibodies. After the membranes were incubated for 1 h at room temperature with the appropriate secondary antibodies, the bands were detected using Immobilon Western chemiluminescent HRP substrate (Millipore).

Cell proliferation assay

The cell proliferation assay was performed as described by Finlay et al. [29] with minor modification. Briefly, RAW 264.7 cells were cultured on 24-well dishes at a density of 1×10^4 cells per well. After 4 days, culture supernatants were aspirated, and cells were stained with 0.3 mL of methylene blue per well. After 30 min at room temperature, plates were inverted briefly to allow most of the stain to drain away. Unbound stain was washed off by immersion in water (3–4 sequential rinses). Plates were air-dried and stored until further processing. Stained cells were dissolved overnight using 1% Sarkosyl (Sigma) in phosphate-buffered saline (0.3 mL/well), and the solution was transferred to a 96-well plate (0.1 mL/well). Absorbance was detected by a plate spectrophotometer at a sample wavelength of 540 nm.

Statistical analyses

Data were presented as means $\pm$ standard deviation. Comparisons between two treatment groups were performed by the two independent samples t-test, and comparisons between three treatment groups were performed by one-way analysis of variance (ANOVA), as well as the least significant difference LSD post-hoc tests for pair-wise groups. Bonferroni corrections for the post-hoc tests were used. All statistical assessments were two-sided and evaluated at the 0.05 level of significance. Statistical analyses were performed using the SPSS 15.0 statistics software (SPSS Inc, Chicago, Illinois, USA).

Results

CO diminished RANKL expression in the synovium of arthritic mice

Given the role of RANKL in inducing osteoclast differentiation [5] and subsequent bone loss [8], we first established an *in vivo* model of arthritis induced by collagen antibody and assessed RANKL expression in the synovium. As shown in Figure 2A, RANKL mRNA was significantly induced in arthritic mice ($p < 0.001$), but was suppressed with CO ($p < 0.001$). Similar effects were observed with RANKL protein (Fig. 2B).

CO treatment increased RAW 264.7 cell proliferation

To determine the mechanism by which CO reduced RANKL expression, we next assessed its effects on RAW 264.7 cells by determining cell number and activated caspase-3 levels. CO slightly increased the total cell number of RAW cells as compared to the control group, which was already considerably high (0.126 vs. 0.112, respectively; Fig. 3A). Analysis of caspase 3 activation as a measure of apoptosis revealed nearly no apoptosis in both the air and CO treatment groups; H_2O_2 significantly induced greater activated caspase 3 levels ($p < 0.001$; Fig. 3B).

Fig. 2. CO diminished RANKL expression in the synovium of arthritic mice. The control group consisted of naïve mice while the collagen antibody-induced arthritic (CAIA) groups were either exposed to room air (CAIA) or air mixed with 250 ppm CO (CAIA+CO). After the synovial membrane extracts were obtained, (A) RANKL mRNA expression relative to GAPDH. Data are represented as mean $\pm$ SD ($n = 3$). (B) RANKL protein expression was also determined in the synovial membrane extracts. The numbers at the bottom of each panel represent the density of the RANKL band after normalization to the internal control, GAPDH.

Fig. 3. CO increased the proliferation of RAW 264.7 cells. (A) RAW 264.7 cells were exposed to air or CO (250 ppm), and total cell number was determined by methylene blue staining. Data are represented as mean $\pm$ SD ($n = 6$). (B) Western blot analysis of activated caspase-3 in RAW 264.7 cells exposed to air, CO (250 ppm) or H_2O_2 (positive control). Numbers at the bottom represent the density of the activated caspase-3 band after normalization to actin, the internal control. Data are represented as mean $\pm$ SD ($n = 3$).

Addition of the PPAR- γ agonist, Troglitazone, reversed the inhibitory effect of CO

The nuclear receptor, PPAR- γ , can regulate osteoclastogenesis by directly regulating c-Fos expression [25]. To determine if the effects of CO on osteoclastogenesis were mediated in part through altered PPAR- γ activation, we next evaluated the effects of the PPAR- γ agonist, Troglitazone, by assessing the number of RAW 264.7 cells expressing TRAP, a mature osteoclast-specific marker [24]. As shown in Figure 4, the number of TRAP-positive cells increased with exposure to air plus RANKL and Troglitazone as compared to the air plus RANKL group ($p=0.021$). In addition, the number of TRAP-positive cells decreased with CO plus RANKL ($p=0.007$), but was increased with the addition of Troglitazone to control levels ($p=0.006$; Fig. 4). Representative images from each treatment group are shown in the right panels.

Fig. 4. Addition of the PPAR γ agonist, Troglitazone, reversed the inhibitory effect of CO on osteoclast differentiation. RAW 264.7 cells were exposed to either air with RANKL (20 ng/mL) or CO (250 ppm) with RANKL in the presence and absence of 0.5 μ M Troglitazone. Data are represented as mean $\pm$ SD (n=3). Representative images are shown in the right panels. (top left) After RANKL stimulation, RAW 264.7 cells aggregated and fused to form a single giant cell (osteoclast), which was surrounded by an actin ring. The red arrow indicates a normal, nonaggregated RAW 264.7 cell. (top right) CO inhibited RANKL-induced differentiation into osteoclasts. (bottom panels) Addition of Troglitazone to RANKL-treated cells exposed to either air or CO resulted in the formation of osteoclasts to a greater degree than that observed in the absence of Troglitazone (top panels).

Discussion

The possible therapeutic applications of CO have been reported in different disease models. For example, Sato et al. [30] reported that CO increased the survival of mouse-to-rat cardiac grafts. Inhalation of CO could also protect mice from death after ischemic lung injury [31]. The protective effects of CO for autoimmune diseases have also been reported [32]. In a murine collagen-induced arthritis model, CORM reduced inflammation, RANKL expression and joint destruction [16]. Furthermore, in RANKL-stimulated RAW 264.7 cells, we previously showed that CO suppressed osteoclastogenesis [23]. Therefore, we postulated that exogenous CO could reduce *in vivo* RANKL production via inhibition of inflammatory response. In the present study, the suppression of RANKL expression by CO was confirmed in a murine model of arthritis. In RANKL-stimulated RAW 264.7 cells, CO slightly increased cell number to a significant level; however, no changes in activated caspase-3 levels were noted. In addition, the effects of CO on RANKL-induced TRAP expression were abrogated by the PPAR- γ agonist, Troglitazone.

Cross-talk between CO and PPAR via heme oxygenase has been observed [33]. Specifically, PPAR γ induces heme oxygenase activity, and heme oxygenase upregulates PPAR γ expression [33]. In addition, CO-mediated PPAR- γ activation in LPS-treated RAW 264.7 cells can be inhibited by CORM-2 [34]. Therefore, we also investigated the role of PPAR- γ in the suppression of RANKL-induced osteoclastogenesis by CO. CO inhibited the effects of RANKL on PPAR- γ expression. Moreover, the PPAR- γ agonist, Troglitazone, could reverse the inhibitory effects of CO on osteoclastogenesis. These results are consistent with a recent study that showed that CO can orchestrate a protective effect through the PPAR- γ , which is crucial for the anti-inflammatory response [35]. Although its effects on adipogenesis activation and osteoblastogenesis repression [27] have been well-characterized, few studies have analyzed the role of PPAR- γ in osteoclastogenesis. Administration of the PPAR- γ agonist, thiazolidinediones (TZDs), induces bone loss [36], and an inhibitory effect of the PPAR- γ ligand in osteoclastogenesis has been reported [37-40]. Furthermore, Wan et al. [27] demonstrated that PPAR- γ and its agonist can enhance osteoclastogenesis *in vivo* and *in*

vitro by maintaining the expression of c-Fos, suggesting that PPAR- γ functions as a direct regulator of c-Fos expression and deficiency of PPAR- γ selectively blocks the c-Fos arm of the RANKL signaling pathways [27]. Taken together, CO may regulate RANKL-induced osteoclastogenesis by deactivating the AP-1 complex, through down-regulating c-Fos by interfering with the PPAR- γ pathway in osteoclast progenitors.

Conclusion

To our knowledge, we have shown for the first time that CO suppresses osteoclastogenesis induced by RANKL through suppressing PPAR- γ in RAW 264.7 cells. These findings suggest CO not only can reduce *in vivo* RANKL expression but also interfere with RANKL-induced osteoclastogenesis. Further studies are necessary to evaluate the possibility of developing CO as a novel anti-resorptive agent to inhibit osteoclastogenesis.

Abbreviations

ANOVA (one-way analysis of variance); ACP5 (acid phosphatase 5); CAIA (collagen antibody-induced arthritis); Calcr (calcitonin receptor); CO (carbon monoxide); CORM-2 (CO releasing molecule-2); IL-1 β (interleukin-1 β); MMP9 (matrix metalloproteinase-9); NFATc1 (nuclear factor of activated T-cells, cytoplasmic 1); PPAR- γ (peroxisome proliferator-activated receptor gamma); RA (rheumatic arthritis); RANKL (receptor activator of NF- κ B ligand); TNF (tumor necrosis factor); TGF- β (transforming growth factor β); TRAP (tartrate resistant acid phosphatase).

Acknowledgements

This study elucidates the mechanism by which carbon monoxide inhibits osteoclastogenesis.

This study shows CO suppresses osteoclast differentiation by inhibiting the RANKL-induced activation of PPAR- γ .

CO not only reduces RANKL expression but also interferes with RANKL-induced osteoclastogenesis.

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Disclosure Statement

None

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