Original Paper

Effect of Carbon Monoxide Donor CORM-2 on Vitamin D₃ Metabolism

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Key Words

CORM-2 • 1,25-Dihydroxyvitamin D₃ • Klotho • FGF23 • Phosphate • Calcium

Abstract

Background/Aims: Carbon monoxide (CO) interferes with cytochrome-dependent cellular functions and acts as gaseous transmitter. CO is released from CO-releasing molecules (CORM) including tricarbonyl-dichlororuthenium (II) dimer (CORM-2), molecules considered for the treatment of several disorders including vascular dysfunction, inflammation, tissue ischemia and organ rejection. Cytochrome P450-sensitive function include formation of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) by renal 25-hydroxyvitamin D₃ 1- α -hydroxylase (Cyp27b1). The enzyme is regulated by PTH, FGF23 and klotho. 1,25(OH)₂D₃ regulates Ca²⁺ and phosphate transport as well as klotho expression. The present study explored, whether CORM-2 influences 1,25(OH)₂D₃ formation and klotho expression. **Methods:** Mice were treated with intravenous CORM-2 (20 mg/kg body weight). Plasma 1,25(OH)₂D₃ and FGF23 concentrations were determined by ELISA, phosphate, calcium and creatinine concentrations by colorimetric methods, transcript levels by quantitative RT-PCR and protein expression by western blotting. Fgf23 mRNA transcript levels were further determined in rat osteosarcoma UMR106 cells without or with prior treatment for 24 hours with 20 μ M CORM-2. **Results:** CORM-2 injection within 24 hours significantly increased FGF23 plasma levels and decreased 1,25(OH)₂D₃ plasma levels, renal Cyp27b1 gene expression as well as renal klotho protein abundance and transcript levels. Moreover, treatment of UMR106 cells with CORM-2 significantly increased Fgf23 transcript levels. **Conclusion:** CO-releasing molecule CORM-2 enhances FGF23 expression and release and decreases klotho expression and 1,25(OH)₂D₃ synthesis.

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Introduction

Carbon monoxide (CO), a gas, which is highly toxic to humans and animals when encountered in higher concentrations, binds rapidly to hemoglobin (Hb), leading to the stable formation of carboxyhemoglobin (COHb) [1]. As a result of CO binding to Hb both O₂ binding at high and O₂ release at low O₂ partial pressure are impaired [1] leading to a reduced oxygen carrying capacity of blood after CO inhalation [2].

Besides its toxic effects at high concentrations, CO is a gaseous transmitter molecule [3]. In mammalian cells CO is generated by the enzyme heme-oxygenase [4]. CO participates in the regulation of a wide variety of cellular functions [5-9] and contributes to the regulation of cell death [10-15]. CO may elicit both vasodilation and vasoconstriction [16, 17]. In addition, CO fosters coagulation and attenuates fibrinolysis *in vitro* and *in vivo* [18, 19]. CO can be released in an organism by the tricarbonyl-dichlororuthenium (II) dimer (CORM-2) [6]. CO-releasing molecules have been suggested as therapeutic option in various pathologies, but the risks of treatment with those molecules have been insufficiently addressed [6, 20].

CO interferes with the function of cytochromes [21-23]. The cytochrome P450 enzyme 25-hydroxyvitamin D₃ 1-alpha-hydroxylase accomplishes formation of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) from its inactive precursor (25-hydroxyvitamin D₃) [24]. The enzyme 25-hydroxyvitamin D₃ 1-alpha-hydroxylase is encoded by the Cyp27b1 gene [24]. 1,25(OH)₂D₃ is inactivated by 1,25-dihydroxyvitamin D₃ 24-hydroxylase, an enzyme encoded by the Cyp24a1 gene [25]. 1,25(OH)₂D₃ is a powerful regulator of CaHPO₄ metabolism and lack of 1,25(OH)₂D₃ leads to rickets and osteomalacia [26]. Reduced levels of 1,25(OH)₂D₃ are further associated with the onset and progression of various diseases such as respiratory infections, cardiovascular disease and cancer [27-30]. 1,25(OH)₂D₃ formation is inhibited by joint action of klotho and FGF23, proteins regulating phosphate homeostasis and implicated in various disorders including arteriosclerosis, cancer metastasis, and chronic kidney disease [31-33]. Furthermore, heme-oxygenase-1 is involved in bone metabolism and inflammation [34, 35].

The present study explored, whether the CO donor tricarbonyl-dichlororuthenium (II) dimer (CORM-2) influences vitamin D₃ metabolism [6, 14]. To this end, vitamin D metabolism was investigated after CORM-2 injection into mice.

Materials and Methods

All animal experiments were conducted according to the German law for the welfare of animals and were approved by local authorities. Male and female BALB/c mice had free access to food (ssniff Spezialdiäten, Soest, Germany) containing 7000 mg/kg phosphorus and to tap drinking water ad libitum. Where indicated, the CO-releasing molecule tricarbonyldichlororuthenium (II) dimer (CORM-2, Sigma Aldrich, Taufkirchen, Germany), solubilized in dimethyl sulfoxide (DMSO, Sigma Aldrich), was injected intravenously (20 mg/kg body weight). Control mice were injected with vehicle (10% DMSO in saline solution).

To determine excretion of phosphate, the mice were placed individually in metabolic cages (Techniplast, Hohenpeissenberg, Germany) for 24-hour urine collection [36, 37]. They were allowed a 2-day habituation period. Subsequently, 24-hour collection of urine was performed. To assure quantitative urine collection, metabolic cages were siliconized, and urine was collected under water-saturated oil [38]. The urinary phosphate concentration was determined colorimetrically utilizing a commercial diagnostic kit (Roche Diagnostics, Mannheim, Germany) [39].

To obtain blood specimens, mice were lightly anaesthetized with isoflurane (Abott, Wiesbaden-Delkenheim, Germany) and about 50 - 200 µl of blood was withdrawn into heparinized capillaries by puncturing the retro-orbital plexus [40]. The plasma concentrations of phosphate and Ca²⁺ were measured by a photometric method (FUJI FDC 3500i, Sysmex, Norsted, Germany). The creatinine concentrations in plasma were measured using a commercial enzymatic kit (creatinine PAP, Labor & Technik, Berlin, Germany, based on the creatininase method). An EIA kit was employed to determine plasma concentrations of 1,25(OH)₂D₃ (IDS, Boldon, UK). Plasma FGF23 (C-Term) concentrations were determined utilizing a commercial ELISA Kit (Immutopics, San Clemente, USA) [41].

After 24 hours of CORM-2 treatment, mice were anesthetized with isoflurane and sacrificed by cervical dislocation. The kidneys were removed and immediately snap frozen in liquid nitrogen [42]. To determine protein expression by western blotting, kidneys were lysed with ice-cold lysis buffer (54.6 mM HEPES; 2.69 mM Na₄P₂O₇; 360 mM NaCl; 10% [vol/vol] Glycerol; 1% [vol/vol] NP40) containing phosphatase and protease inhibitors (Complete mini, Roche, Mannheim, Germany). Homogenates were clarified by centrifugation at 12000 rpm for 20 min [43]. Total protein was separated by SDS-PAGE (8% Tris-Glycine), transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany), blocked for 1 h in blocking buffer (5% fat-free milk in Tris-buffered saline (TBS) containing 0.1% Tween (TBS-T)), and incubated overnight with an anti-klotho antibody (1:1000 in 5% fat-free milk in TBS-T, KM2076 (Rat), kindly provided by Kyowa Hakko Kirin Co. Ltd, Japan). After incubation with an anti-rat secondary antibody (1:2000 in 5% fat-free milk in TBS-T, Cell Signaling), the bands were visualized with enhanced chemiluminescence according to the manufacturer's instructions. Homogenates were also probed with a primary Gapdh antibody (1:2000 in 5% BSA in TBS-T, Cell Signaling) as a loading control. Densitometric analysis of klotho and Gapdh was performed using Quantity One software (Bio-Rad Laboratories, München, Germany).

Rat osteosarcoma UMR106 cells were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/l glucose (PAA Laboratories, Cölbe, Germany), supplemented with 2 mM L-glutamine (PAA Laboratories, Cölbe, Germany), 10% fetal bovine serum FBS (Gibco, Life Technologies, Darmstadt, Germany), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Life Technologies, Darmstadt, Germany). Cells were treated for 24 hours with 20 µM CORM-2 (Sigma-Aldrich, Taufkirchen, Germany) dissolved in DMSO (Sigma-Aldrich, Taufkirchen, Germany). Equal amounts of vehicle were used as control.

Total RNA was isolated from kidney samples and from UMR106 cells by using Trifast Reagent (Peqlab, Erlangen, Germany) according to the manufacturer's instructions [44]. Reverse transcription of 2 µg RNA was performed using oligo(dT)₁₂₋₁₈ primers (Invitrogen, Darmstadt, Germany) and SuperScriptIII Reverse Transcriptase (Invitrogen, Darmstadt, Germany). cDNA samples were treated with RNaseH (Invitrogen, Darmstadt, Germany). Quantitative real-time PCR was performed with the iCycler iQ^{TM} Real-Time PCR Detection System (Bio-Rad Laboratories, München, Germany) and iQ^{TM} Sybr Green Supermix (Bio-Rad Laboratories, München, Germany) according to the manufacturer's instructions. The following mouse primers were used (5'→3' orientation) for quantitative RT-PCR measurements:

Cyp24a1 fw: GTGAAGCGTGCGCCAAAAG; *Cyp24a1* rev: CTCACGTCGGTCATCAGC;
Cyp27b1 fw: CAGTTTACGTTGCCGACCCTA; *Cyp27b1* rev: GGACAGTGACTTTCTTGTCGC;
Gapdh fw: AGGTCGGTGTGAACGGATTG; *Gapdh* rev: TGTAGACCATGTAGTTGAGGTCA;
Klotho fw: CCCTGTGACTTTGCTTGGG; *Klotho* rev: CCCACAGATAGACATTCGGGT.

The following rat primers were used (5'→3' orientation) for quantitative RT-PCR measurements:

Fgf23 fw: TTGGATCGTATCACTTCAGC; *Fgf23* rev: TGCTTCGGTGACAGGTAG;
Gapdh fw: GGCAAGTTCAATGGCACAGT; *Gapdh* rev: TGGTGAAGACGCCAGTAGACTC.

The specificity of the PCR products was confirmed by analysis of the melting curves and in addition by agarose gel electrophoresis. All PCRs were performed in duplicate, and mRNA fold changes were calculated by the 2^{-ΔΔCt} method using Gapdh as internal reference.

Data are expressed as arithmetic means ± SEM, and n represents the number of independent experiments. Statistical analysis was made using unpaired Student *t*-test or Mann-Whitney test. Only differences with *p* < 0.05 were considered statistically significant.

Results

To investigate the effects of the tricarbonyl-dichlororuthenium (II) dimer (CORM-2) on vitamin D metabolism, the CO-releasing molecule CORM-2 was administered to mice by intravenous injection. For comparison, equal amounts of DMSO vehicle was injected into control mice. CORM-2 releases a controlled amount of CO in biological systems [6]. CORM-2 treatment of mice for 24 hours led to a statistically significant decrease of plasma 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) concentration as compared to the control treated mice (Fig. 1). The decrease of plasma 1,25(OH)₂D₃ concentrations following CORM-2

Fig. 1. Arithmetic means $\pm$ SEM ($n = 8$) of plasma calcitriol concentration (pmol/l) in mice 24 hours after the injection of vehicle control (ctr, white bar) or the injection of 20 mg/kg body weight CORM-2 (CORM-2, black bar). **($p < 0.01$) indicates statistically significant difference from vehicle treated mice.

Fig. 2. Arithmetic means $\pm$ SEM ($n = 7-8$) of *Cyp27b1* (A) and *Cyp24a1* (B) mRNA expression (arbitrary units) in kidney tissues from mice 24 hours after the injection of vehicle control (ctr, white bars) or the injection of 20 mg/kg body weight CORM-2 (CORM-2, black bars). ***($p < 0.001$) indicates statistically significant difference from vehicle treated mice.

treatment was accompanied by a statistically significant decrease of renal transcript levels of *Cyp27b1*, the gene encoding 25-hydroxyvitamin D₃ 1- α -hydroxylase, a cytochrome P450-dependent enzyme producing 1,25(OH)₂D₃ (Fig. 2A). Transcript levels of *Cyp24a1* encoding the 1,25(OH)₂D₃ inactivating 1,25-dihydroxyvitamin D₃ 24-hydroxylase tended to increase following CORM-2 treatment, an effect, however, not reaching statistical significance (Fig. 2B). Despite reduced 1,25(OH)₂D₃ levels, no significant difference was observed in the plasma Ca²⁺ concentrations between the control treated mice (8.90 ± 0.17 mg/dl, $n = 12$) and CORM-2 treated mice (9.09 ± 0.13 mg/dl, $n = 12$). Similarly, plasma phosphate concentrations were not significantly different between control treated mice (6.75 ± 0.36 mg/dl, $n = 12$) and CORM-2 treated mice (6.76 ± 0.37 mg/dl, $n = 12$). Furthermore, the urinary phosphate excretion was not significantly different between control treated mice (2.34 ± 0.36 μ mol/24h/[g]BW, $n = 12$) and CORM-2 treated mice (2.65 ± 0.42 μ mol/24h/[g]BW, $n = 12$). In addition, 12 hours after the injection of CORM-2, plasma creatinine concentrations were not significantly different between CORM-2 treated mice (0.383 ± 0.040 mg/dl, $n = 5$) and control treated mice (0.300 ± 0.038 mg/dl, $n = 5$).

CORM-2 injection was, however, followed by an increase of plasma FGF23 concentration. As shown in Fig. 3A, the plasma FGF23 (C-Term) levels were significantly higher in mice receiving CORM-2 treatment than in control treated mice. To investigate, whether CORM-2 could directly affect FGF23 production, rat osteosarcoma UMR106 cells were treated for 24 hours with DMSO as control or with 20 μ M CORM-2 (Fig. 3B). As a result, CORM-2 induced a statistically significant increase in Fgf23 mRNA expression in UMR106 cells as compared to the vehicle treated UMR106 cells.

Renal *Klotho* mRNA levels determined by quantitative RT-PCR, were significantly lower in mice following CORM-2 treatment than in control treated mice (Fig. 4A). According to

Fig. 3. (A) Arithmetic means $\pm$ SEM ($n = 12$) of plasma FGF23 (C-Term) concentration (pg/ml) in mice 24 hours after the injection of vehicle control (ctr; white bar) or the injection of 20 mg/kg body weight CORM-2 (CORM-2, black bar). *($p < 0.05$) indicates statistically significant difference from vehicle treated mice. (B) Arithmetic means $\pm$ SEM ($n = 10$; arbitrary units) of *Fgf23* mRNA expression in UMR106 cells following a 24 hours treatment with vehicle alone (ctr; white bar) or with 20 μ M CORM-2 (CORM-2, black bar). *($p < 0.05$) indicates statistically significant difference from UMR106 cells treated with vehicle alone.

Fig. 4. (A) Arithmetic means $\pm$ SEM ($n = 7-8$) of *Klotho* mRNA expression (arbitrary units) in kidney tissues from mice 24 hours after the injection of vehicle control (ctr; white bar) or the injection of 20 mg/kg body weight CORM-2 (CORM-2, black bar). (B) Representative original western blots showing *Klotho*/*Gapdh* protein abundance in kidney tissues from mice following the injection of vehicle control (ctr) or the injection of 20 mg/kg body weight CORM-2 (CORM-2). (C) Arithmetic means $\pm$ SEM ($n = 7$) of *Klotho*/*Gapdh* protein ratio in kidney tissues from mice 24 hours after the injection of vehicle control (ctr; white bar) or the injection of 20 mg/kg body weight CORM-2 (CORM-2, black bar). ***($p < 0.001$) indicates statistically significant difference from vehicle treated mice.

western blotting, the renal abundance of *klotho* protein was again significantly lower in CORM-2 treated mice than in control treated mice (Fig. 4B,C).

Discussion

The present study discloses that the CO donor tricarbonyl-dichlororuthenium (II) dimer (CORM-2) decreases renal transcript levels of *Cyp27b1*, the gene encoding the 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃)-generating 25-hydroxyvitamin D₃ 1- α -

hydroxylase. Accordingly, CORM-2 decreases the plasma 1,25(OH)₂D₃ level. The observations further reveal that CORM-2 decreases renal *klotho* expression, increases plasma FGF23 levels and enhances FGF23 expression in rat osteosarcoma UMR106 cells.

FGF23 is mainly produced and released by osteoblasts/osteocytes [45]. The up-regulation of FGF23 plasma levels and mRNA expression in UMR106 cells may thus point to a direct effect of CORM-2 on FGF23 release from bone. We cannot rule out effects of CORM-2 indirectly modifying FGF23 release. As FGF23 release is stimulated by 1,25(OH)₂D₃ [45], the up-regulation of FGF23 release cannot be explained by the decline of 1,25(OH)₂D₃ plasma levels. As FGF23 downregulates 1- α -hydroxylase [45], the increased FGF23 plasma levels could, however, contribute to or even account for the decreased 1,25(OH)₂D₃ formation following CORM-2 treatment. FGF23 modifies 1,25(OH)₂D₃ plasma levels by inhibiting formation rather than stimulating 1,25(OH)₂D₃ degradation [46]. The inhibitory effect on 1,25(OH)₂D₃ formation could further be due to down-regulation of *Cyp27b1* (Fig. 2) and/or binding of CO to cytochrome P450 with subsequent impairment of 25-hydroxyvitamin D₃ 1- α -hydroxylase activity [24]. The downregulation of *klotho* would, however, be expected to attenuate the effect of FGF23 on 25-hydroxyvitamin D₃ 1- α -hydroxylase activity [45, 47].

CO increases cellular oxygen consumption, reduces mitochondrial respiratory capacity and increases VEGF production via HIF-1 α , which could possibly contribute to the regulation of FGF23 by CORM-2 [48, 49]. Along those lines production of FGF23, *Cyp24a1* and *Cyp27b1* expression as well as 1,25(OH)₂ vitamin D₃ plasma levels are modified by iron deficiency, an effect associated with tissue hypoxia [50, 51].

As 1,25(OH)₂D₃ stimulates the expression of *klotho* [45], the decrease of 1,25(OH)₂D₃ formation could contribute to or even account for the decrease of *klotho* expression following CORM-2 treatment. The regulation of *Klotho* mRNA expression is vitamin D₃ receptor-mediated, and vitamin D₃ response elements (VDREs) in both mouse and human *klotho* genes have been identified [52]. Beyond that, a direct toxic effect of the ruthenium based carrier molecule of CORM-2 on the kidney cannot be ruled out [20]. CORM-2 did, however, not significantly affect plasma creatinine, calcium and phosphate concentrations or urinary phosphate excretion.

The influence of CORM-2 described here may contribute to the known effects of CORM-2. Several vasculoprotective effects of lower dosages of CORM-2 have been described and low dosages of CO are associated with nephroprotection [53]. However, increased endogenous carbon monoxide production may be involved in diabetes induced endothelial dysfunction [54]. Similarly, *klotho* is decreased in the db/db, streptozotocin diabetes models and human diabetic nephropathy [55-57]. Heme oxygenase derived endogenous CO promotes endothelial dysfunction in hypertensive rats, and high levels of CO could impair nitric oxide release [58]. Blockage of the nitric oxide synthase also leads to reduced *klotho* expression [59].

Biological effects of 1,25(OH)₂D₃ are mainly mediated by activation of the vitamin D₃ receptor (VDR) [60, 61]. Low 1,25-dihydroxyvitamin D₃ levels may lead to demineralization of bone [26] and in addition play a role in the development and progression of multiple chronic diseases such as cancer, infections, and cardiovascular disorders [27-30].

The present observations may hint at effects of smoking, which leads to massive formation of CO [62, 63]. CO binds rapidly to hemoglobin (Hb), leading to the stable formation of carboxyhemoglobin (COHb) [2]. Similar to 1,25(OH)₂D₃ formation after CORM-2 treatment, smoking is associated with reduced vitamin D₃ levels without changes in serum calcium [64]. Furthermore, history of tobacco smoking is associated with elevated FGF23 levels in CKD patients [65]. Tobacco smoke plays a decisive role in the pathogenesis of lung cancer [66], chronic obstructive pulmonary disease (COPD) [66] and cardiovascular disease [67]. The underlying mechanisms are incompletely understood [68], but may, at least in theory, involve altered action of 1,25(OH)₂D₃, *klotho* or FGF23. Both, COPD and smoking, are associated with an increased carbon monoxide load and risk of vitamin D₃ deficiency [69, 70]. 1,25(OH)₂D₃ may be a potent regulator of several signaling pathways, which are involved in oncogenic transformation [25, 61, 71]. Secreted *klotho* functions as a humoral factor and regulates the

activity of multiple growth factors, including insulin/insulin-like growth factor-1 (IGF-1) [72], Wnt [73], and TGF- β 1 [74]. Soluble klotho has been identified as a potent inhibitor of the insulin/IGF-1 pathways in cancer cells [75, 76]. Klotho inhibits lung cancer cell growth and klotho expression is a predictor of a favourable outcome in lung cancer patients [77, 78]. Future studies will be required to define the role of decreased 1,25(OH)₂D₃ and klotho formation in the untoward effects of CO.

Conclusion

CORM-2 leads to a decrease of 25-hydroxyvitamin D₃ 1-alpha-hydroxylase expression and activity resulting in decreased formation of 1,25(OH)₂D₃ and klotho expression. Moreover, CORM-2 increases the formation of FGF23. The observations disclose an impact of CO-releasing molecules on mineral metabolism.

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