Carbon Monoxide (CO) Is a Novel Inhibitor of Connexin Hemichannels*

Received for publication, August 5, 2014, and in revised form, November 7, 2014. Published, JBC Papers in Press, November 10, 2014, DOI 10.1074/jbc.M114.602243

Carmen G. León-Paravic†, Vania A. Figueroa‡, Diego J. Guzmán‡, Carlos F. Valderrama‡, Antonio A. Vallejos†, Mariana C. Fiori§, Guillermo A. Altenberg§, Luis Reuss§, and Mauricio A. Retamal‡,§,†

From the †Centro de Fisiología Celular e Integrativa, Facultad de Medicina, Clínica Alemana Universidad del Desarrollo, Santiago, Chile 7690000 and the ‡Department of Cell Physiology and Molecular Biophysics, and Center for Membrane Protein Research, Texas Tech University Health Sciences Center, Lubbock, Texas 79430

Background: Carbon monoxide and connexin hemichannels are involved in several physiological and pathological processes.

Results: Carbon monoxide inhibits Cx43 and Cx46 hemichannel opening.

Conclusion: Connexin hemichannels are modulated by gaseous transmitters.

Significance: Our observations will help understand the effects of CO and CO donors in pathological conditions.

Hemichannels (HCs) are hexamers of connexins that can form gap-junction channels at points of cell contacts or “free HCs” at non-contacting regions. HCs are involved in paracrine and autocrine cell signaling, and under pathological conditions may induce and/or accelerate cell death. Therefore, studies of HC regulation are of great significance. Nitric oxide affects the activity of Cx43 and Cx46 HCs, whereas carbon monoxide (CO), another gaseous transmitter, modulates the activity of several ion channels, but its effect on HCs has not been explored. We studied the effect of CO donors (CORMs) on Cx46 HCs expressed in Xenopus laevis oocytes using two-electrode voltage clamp and on Cx43 and Cx46 expressed in HeLa cells using a dye-uptake technique. CORM-2 inhibited Cx46 HC currents in a concentration-dependent manner. The C-terminal domain and intracellular Cys were not necessary for the inhibition. The effect of CORM-2 was not prevented by guanyllyl-cyclase, protein kinase G, or thioredoxin inhibitors, and was not due to endocytosis of HCs. However, the effect of CORM-2 was reversed by reducing agents that act extracellularly. Additionally, CO inhibited dye uptake of HeLa cells expressing Cx43 or Cx46, and MCF-7 cells, which endogenously express Cx43 and Cx46. Because CORM-2 carboxylates Cx46 in vitro and induces conformational changes, a direct effect of that CO on Cx46 is possible. The inhibition of HCs could help to understand some of the biological actions of CO in physiological and pathological conditions.

Hemichannels (HCs) are hexamers of connexins thought to be normally closed, preventing cell losses of important metabolites such as ATP and glutathione, as well as massive entry of Ca²⁺. However, recent studies show that HCs undergo brief openings under physiological conditions; HC opening has been observed in ephaptic communication in the vertebrate retina, during the spread of Ca²⁺ waves, memory consolidation in the amygdala, and neurotransmitter release. It is also known that HCs with high activity (leaky HCs) are observed in several diseases where it contributes to cell damage or death. Overall, these studies support the idea that brief HC openings are compatible with life and are relevant in physiological conditions; however, uncontrolled HC opening is detrimental.

The mechanisms of HC gating vary among connexin isoforms. Phosphorylation, protease cleavage, and membrane potential are well known factors. Recently, it has been proposed that changes in redox potential are also important modulators of Cx43 and Cx46 HCs; for example, nitric oxide (NO) induces the S-nitrosylation of Cx43 intracellular Cys and causes HC opening. NO is probably the most studied of the gas transmitters, which also include carbon monoxide (CO) and hydrogen sulfide (H₂S).

Under physiological conditions, CO is a byproduct and a substrate of decomposition of heme groups that results from the work of heme oxygenases types I (HO-I) and II (HO-II); HO-I is inducible, whereas HO-II is constitutive. The actions of CO involve two pathways: (i) activation of guanylate cyclase with production of cGMP, and (ii) carboxylation of amino acids such as proline, threonine, lysine, and arginine. The physiological importance of CO is supported by the fact that cell cultures of HO-I knock-out mice show elevated concentrations of free radicals, and the few animals that reach birth (less than 5%) present serious disturbances in liver function and high mortality. These results suggest that CO is vital for the development and function of several tissues. Abnormal endogenous CO effects have been demonstrated in several diseases, and
exogenous CO administration has potential value in the treatment of inflammation, sepsis, lung injury, cardiovascular diseases, transplantation, and cancer (18, 19). Despite its importance in health and disease, the molecular mechanism of action of CO is not well understood.

As mentioned above, connexin HCs are involved in a plethora of cellular functions, both under physiological and pathological conditions. Cx46 has gained considerable attention lately as a potential target for cancer treatment (20, 21) because it has been suggested that Cx46 helps cancer cells survive in hypoxic conditions (20). Because of its potential role of Cx46 in cancer biology and its sensitivity to gaseous transmitters such as NO (15), we decided to evaluate the possibility that Cx46 HCs are affected by HO-1 effectors (CO or guanylyl cyclase signaling pathways). To test this hypothesis, we determined the effects of the CO donor CORM-2 on HC activity. We studied Cx46 HC currents in X. laevis oocytes using two-electrode voltage-clamp, and dye uptake through Cx46 and Cx46 HCs in HeLa cells. We found that CORM-2 produced a major reduction of Cx46 HC currents, an effect independent of the CO guanylyl cyclase/protein kinase G (PKG) signaling pathway, but associated to Cx46 carboxylation, suggesting that CO has a direct effect on the HCs.

MATERIALS AND METHODS

Chemicals—Tricarbonyldichlororuthenium(II) dimer (CORM-2), tricarbonylchloro(glycinato)ruthenium(II) (CORM-3), Na2SO4 [H2BO3] (CORM-A1), β-mercaptoethanol (β-ME), dithiothreitol (DTT), KT5823, 1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), and MG132 were purchased from Sigma, and 4′,6-diamidino-2-phenylindole (DAPI) from Invitrogen. The chemicals—

Plasmid Engineering—Details on the wild-type Cx46 and mutants were previously described (15). The rat Cx46 in pSP64T plasmid was obtained from Dr. Lisa Ebihara (Rosalind Franklin University of Medicine and Science), and site-directed mutagenesis was used to generate the Cx46-C3A and Cx46ΔCT mutants. In Cx46-C3A the intracellular Cys-218, Cys-283, and Cys-321 were substituted with Ala. Cx46 mutants. In Cx46-C3A the intracellular Cys-218, Cys-283, and Cys-321 were substituted with Ala. Cx46

Expression and Purification of Cx46—A synthetic gene coding for Cx46 followed by a tobacco etch virus protease cleavage sequence and a poly-His tag (His6) was expressed in Escherichia coli. This DNA, optimized for E. coli expression, was subcloned into the pQE60 plasmid, and expression was carried out in XL10-Gold cells grown in a modified M9 medium. After removal of the His tag, purified Cx46 was isolated by gel-filtration chromatography on a Superdex 200HR (w/w). After removal of the His tag, purified Cx46 was isolated by gel-filtration chromatography on a Superdex 200HR column.

Protein Carbonyl Detection—Protein samples were derivatized by the reaction between 2,4-dinitrophenylhydrazine and protein carbonyls. Formation of a Schiff base produces the corrolated by the reaction between 2,4-dinitrophenylhydrazine and protein carbonyls. Formation of a Schiff base produces the corronat product. A DNA, optimized for E. coli expression, was subcloned into the pQE60 plasmid, and expression was carried out in XL10-Gold cells grown in a modified M9 medium. The purification procedure was as previously described for Cx26, using a combination of metal affinity (Talon Co2+ column, Talon Superflow, Clontech) and gel-filtration chromatography (Superdex 200HR 10/300 GL column, GE Healthcare) (23). The tag was removed by incubation with tobacco etch virus protease for 12 h, at a Cx46/tobacco etch virus protease ratio of 1:15 (w/w). After removal of the His tag, purified Cx46 was isolated by gel-filtration chromatography on a Superdex 200HR column.

Trp fluorescence measurements—Trp fluorescence was measured on a Jasco spectrophurometer model FP-6300. Samples containing purified Cx46 were excited at 295 nm and fluorescence emission was measured between the wavelengths of 300–500 nm.
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of 310 and 500 nm. The measurements were carried out at room temperature.

Detection of CO Content in CO-depleted CORM-2 Solution—For the determination of the content of CO in solution, Hb was dissolved to a concentration of 1 mg/ml in ND96 (control) or in ND96 supplemented with 100 μM fresh CORM-2 or CO-depleted CORM-2 solutions. The solutions were supplemented with 0.1% sodium dithionite to deoxygenate them, to allow CO binding to Hb to form carboxyhemoglobin. The content of carboxyhemoglobin was assessed from the changes of the absorbance at 540 nm (24).

Immunofluorescence—MCF-7 cells grown in glass coverslips were fixed by incubation in 4% paraformaldehyde for 30 min, and incubated with PBS, 1% Triton for an additional 15 min. The cells were then incubated with normal goat serum (Santa Cruz, number SC-2043) for 1 h. After overnight incubation with the polyclonal primary antibodies against Cx43 (Invitrogen, number 710700) or Cx46 (Santa Cruz, number M-127) diluted in normal goat serum, the coverslips were washed with PBS and incubated with a secondary antibody (goat anti-rabbit, Thermo Scientific) for 1 h. Cx43 and Cx46 immunolocalization was determined in an epifluorescence-inverted microscope (Nikon, Eclipse Ti).

Statistical Analysis—Results are expressed as mean ± S.E. and n refers to the number of independent experiments. For statistical analyses, each treatment was compared with its respective control, and significance was determined using a one-way analysis of variance or paired Student’s t test, as appropriate. Differences were considered significant at p < 0.05.

RESULTS

CORMs Inhibits Cx46 Hemichannel Currents—Because Cx46 is responsive to changes in redox potential (15) and CO modulates physiological and pathological processes by mechanisms that include effects on ion channels (25), we tested whether CO modulates rat Cx46 HC currents. With repeated depolarizing pulses from −60 to +60 mV for 2.5 s, with 2.5-s intervals, HC currents increase progressively until they reach a stable maximum. We previously described this phenomenon and called it “facilitation” (26). Application of 100 μM CORM-2 once the currents stabilized caused inhibition of Cx46 HC currents (Fig. 1A) with a time constant (τ) of 29 s. This concentration of CORM-2 has been previously used in studies on other ion channels (27–30). To study the voltage dependence of the CORM-2 inhibition, oocytes were exposed to 100 μM CORM-2 for 3 min (to ensure a maximal effect) and HC currents were recorded using a protocol wherein oocytes were held at −60 mV and then exposed for 15 s to voltages ranging from −60 to +60 mV in 10 mV steps. After each pulse, the membrane potential was held for 10 s at −60 mV (15). Under control conditions, Cx46 HC currents activated slowly at voltages over +10 mV, showing a clear tail current when the voltage returned to −60 mV (Fig. 1B, lower panel). At +60 mV the maximum current was 12.0 ± 1.1 μA. These results are consistent with our previous studies (15).

After the 3-min exposure to 100 μM CORM-2, Cx46 HC currents were inhibited by 73 ± 4% (Fig. 1, B and D). Oocytes injected only with antisense for Cx38 show a small current (0.37 ± 0.17 μA) that did not change after CORM-2 application (0.39 ± 0.14 μA) (Fig. 1B, upper panel). Fig. 1C shows that increasing CORM-2 concentrations progressively decreased the HC currents with an IC50 of 3.4 μM. Unfortunately, the voltage clamp was difficult to maintain at higher concentrations of CORM-2. We do not know whether this is the result of alterations in plasma membrane properties or other effects. The V50 values of the whole-cell currents determined by fitting to the Boltzmann equation were 39 ± 1 mV under control conditions and 44 ± 10 mV after CORM-2. The lack of a statistically significant difference indicates that CORM-2 reduces either the number of Cx46 HCs opened by depolarization, and/or the single-channel conductance, without changing voltage sensitivity. Exposure to 100 μM CO-depleted CORM-2 solution (see “Materials and Methods”) increased Cx46 HC currents by 49 ± 17% (Fig. 1D). The CO-depleted CORM-2 solution did not react with Hb to form Hb-CO, whereas fresh CORM-2 did (Fig. 2). These results indicate that CORM-2 was effectively depleted of CO by overnight incubation at 37 °C. The absence of Cx46 HC inhibition by CO-depleted CORM-2 suggests that the inhibitory effect of CORM-2 is due to CO, and not to another CORM-2 product. We did not explore the origin of
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FIGURE 2. Detection of CO release from CORM-2 using a Hb-based assay. Hb (1 mg) was dissolved in 1 ml of solution containing fresh CORM-2 (100 μM) (CO+), its vehicle (ethanol, 1:1000) (OH), or a CO-depleted CORM-2 solution (100 μM) (CO−). The solutions were supplemented with 0.1% sodium dithionite to deoxygenate them and allow CO binding to the Hb to form carboxyhemoglobin (CO-Hb). The resulting mixture was maintained at room temperature for 3 min and then the CO-Hb content was assessed from the changes in absorbance at 540 nm (n = 8 for each condition). Data are mean ± S.E.; ***, denote p < 0.001.

activation of Cx46 HCs by CO-depleted CORM-2; it may result from effects of products different from CO. In agreement with the results above, addition of 5.6 mg/ml of hemoglobin (a CO scavenger) to the bath solution before the addition of fresh CORM-2 prevented the current inhibition by CORM-2 (Δ = −6 ± 2%) (Fig. 1D). A clear inhibition of Cx46 HCs currents was also induced by CORM-3 and CORM-A1 (77 ± 3.9 and 39 ± 7.4% inhibition, respectively). (Fig. 1D). These data strongly support the conclusion that the HC current inhibition by CORM-2 is mediated by released CO.

The C-terminal Domain of Cx46 Is Not Involved in Hemichannel Inhibition by CORM-2—Because the C-terminal domains of Cx46 and other connexin isoforms have a regulatory role, we studied the effect of CORM-2 on HCs formed by Cx46ΔCT, a mutant with deletion of the CT domain. Under control conditions, Cx46ΔCT HCs display outward currents activated at positive voltages and current inactivation at voltages over +50 mV (Fig. 3A, left record). After a 3-min exposure to 100 μM CORM-2 HC, the currents were reduced by 72 ± 7%, without changes in the activation voltage and current relaxation (Fig. 3A, right record) (n = 8). Similar to the effect of CORM-2 over wild-type Cx46 HCs, the V50 of Cx46ΔCT HC did not change (18 ± 2 versus 17 ± 7 mV before and after CORM-2, respectively) (Fig. 3B). These results show that the C-terminal domain of Cx46 is not necessary for the reduction of HC current by CORM-2.

The Reversibility of the Inhibition by CORM-2 Is Greatly Enhanced by Reducing Agents—Because Cys residues have a critical role in the CO sensitivity of BK(Ca) channels (31) and CO modulates the redox balance in several cell types (16), we critical role in the CO sensitivity of BK(Ca) channels (31) and studied the role of Cx46 Cys on HC inhibition by CO. Cx46 has CO modulates the redox balance in several cell types (16), we critical role in the CO sensitivity of BK(Ca) channels (31) and studied the role of Cx46 Cys on HC inhibition by CO. Cx46 has critical role in the CO sensitivity of BK(Ca) channels (31) and studied the role of Cx46 Cys on HC inhibition by CO. Cx46 has

FIGURE 3. The C-terminal domain of Cx46 is not involved in CORM-2 hemichannel inhibition. A, representative Cx46ΔCT (C-terminal domain deletion) HC current records under control conditions (left-hand records) and after exposure to 100 μM CORM-2 (right-hand records) (n = 8 for each condition). B, I/V relationship. Currents at the end of each pulse were normalized to the maximal current obtained at +60 mV under control conditions, and the I/V relationship was fitted with a Bolzmann equation. Data are presented as mean ± S.E. (n = 8).

FIGURE 4. Cx46 hemichannel inhibition by CORM-2 is modulated by reducing agents. A, effect of CORM-2 on HCs formed by Cx46ΔCT (Cx46 without intracellular Cys). Currents at the end of each pulse were normalized to the maximal current obtained at ±60 mV under control conditions, and the I/V relationship was fitted with a Bolzmann equation. Exposure to 100 μM CORM-2 was for 3 min. Data are mean ± S.E. (n = 8). B, reversal by reducing agents of the Cx46 HC inhibition by CORM-2. Oocytes were exposed to 100 μM CORM-2 for 3 min and then to 5 mM β-ME (n = 6), 1 mM DTT (n = 6), or 5 mM extracellular reduced glutathione (GSH) (n = 8) for 1 min. GSH was injected to a calculated final concentration of 5 mM (intracellular reduced glutathione, GSHi) (n = 6). Currents were normalized to the control value and are expressed as mean ± S.E. *** indicates p < 0.001 compared with CORM-2. C, effect of CORM-2 on HCs formed by Cx46ΔCT. Currents at the end of each pulse were normalized to the maximal current obtained at ±60 mV under control conditions, and the I/V relationship was fitted with a Bolzmann equation. Exposure to 100 μM CORM-2 was for 3 min. Data are mean ± S.E. (n = 11). D, oocytes expressing Cx46 or Cx46ΔCT were exposed to 100 μM CORM-2 for 3 min, followed by washing with ND96 (1 ml/min for 3 min). Currents measured at ±60 mV were normalized to the control value in the absence of CORM-2. Data are mean ± S.E. (n = 5 for each time point).

Fig. 4D shows that the inhibition of Cx46 HC by exposure to 100 μM CORM-2 for 3 min is reversible. Following extensive washing with ND96, Cx46 HCs currents displayed a slow rate of recovery over time. Linear regression analysis showed a recovery of 0.007 ± 0.002 nA/min for the Cx46 HC currents, whereas Cx46ΔCT HC currents recovered twice as fast (0.014 ± 0.003 nA/min). This recovery is very slow, and may be the result of insertion of non-oxidized Cx46 at the plasma membrane.
and/or decarbonylation of Cx46 HCs that occurs naturally on oocytes.

Carbonylation is a potential mechanism for the inhibitory effect of CORM-2 (32). Carbonylation induced by CO can be reversed by reducing agents and CO may induce secondary Cys carbonylation in response to lipid peroxidation products, a process that can be sensitive to reducing agents (33, 34). Therefore, we tested whether the inhibition of Cx46 HCs currents by exposure to 100 \( \mu \)M CORM-2 for 3 min was reversed by \( \beta \)-ME or dithiothreitol (DTT). After 1-min exposure to 5 mM \( \beta \)-ME or 1 mM DTT (reducing agents that permeate the plasma membrane), the inhibition of Cx46 HC currents by CORM-2 was reversed to 98 ± 0.1 and 102 ± 1.4% of the control value, respectively. Addition of 5 mM reduced glutathione (GSH, a reducing agent that does not permeate the plasma membrane) also recovered the Cx46 HCs currents inhibited by CORM-2 (to 98 ± 1.9% of the control value). However, when GSH was injected into the oocytes (5 mM estimated final concentration), the recovery of Cx46 HCs currents was minimal (Fig. 4B). Because CO-inhibition present in Cx46-C3A HCs was strongly affected by extracellular reducing agents, and secondary carbonylation of Cys can occur in response to lipid peroxidation products (34), we tested the role of the 6 extracellular Cys in the inhibition of HCs by CORM-2. In HCs formed by a Cx46 where all Cys were replaced with Ala (Cx46-CL) CORM-2 produced only a 25 ± 3.9% inhibition (Fig. 4C). Together, these data indicate that the HC inhibition by CO requires the presence of one or more extracellular Cys.

Guanonyl Cyclase, PKG, and Proteasome Pathways Are Not Involved in the CORM-2 Effect—It has been reported that CO activates guanylyl cyclase (18), which elevates the cGMP intracellular concentration with the consequent PKG activation. Because Cx46 is a phosphoprotein, an involvement of guanylyl cyclase and PKG in the effect of CORM-2 is possible. However, pre-incubation for 45 min with 2 \( \mu \)M KT5823 (a specific PKG inhibitor) or 50 \( \mu \)M ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quin oxalin-1-one, a specific guanylyl cyclase inhibitor) (27, 28) did not affect the current inhibition induced by 100 \( \mu \)M CORM-2 (Fig. 5A). It is also known that oxidized proteins can traffic to proteasomes and be degraded (35), which could account for the decrease in Cx46 HC currents by CORM-2 as a result of a loss of HCs from the plasma membrane. Fig. 5A shows that the inhibition of Cx46 HC currents by CORM-2 in oocytes treated with 10 \( \mu \)M MG132 (a proteasome inhibitor) (32) was 78 ± 10%, a value indistinguishable from that in oocytes treated with CORM-2 alone. Because decarbonylation depends on reducing agents acting by a redox-thioredoxin-dependent mechanism (33, 34), we explored the effects of the thioredoxin reductase inhibitor auranofin on the inhibition of Cx46 HC currents by CORM-2. Oocytes were preincubated with 60 \( \mu \)M auranofin for 30 min and then exposed to 100 \( \mu \)M CORM-2 for 3 min in the continued presence of auranofin. Under these conditions, CORM-2 inhibited Cx46 HC currents by ~60%, indicating that thioredoxin reductase is not directly involved in the HC current recovery induced by reducing agents (Fig. 5A). These data suggest that CO-induced inhibition is not due to HC internalization or activation of a cGMP-dependent pathway.

CORM-2 Inhibits Human Cx43 and Cx46 Hemichannels in Mammalian Cultured Cells—Because signaling and protein-protein interactions are different in frog oocytes and mammalian cells, it is important to demonstrate that the response of HCs to CO is preserved in mammalian cells. To this end, we studied the effect of CORM-2 on HeLa and MCF-7 cells expressing human Cx43 and/or Cx46 using a dye-uptake technique. Under control conditions, Cx46-transfected HeLa cells present a prominent DAPI uptake when exposed to 5 \( \mu \)M of the dye (rate of uptake = 64 ± 8 AU/min) (Fig. 6A). After adding 1 \( \mu \)M CORM-2 (a concentration that shows the maximum effect in Cx43 and Cx46, Fig. 6, B and C), the uptake was reduced drastically (to 23 ± 5 AU/min). Comparable results were obtained in HeLa cells expressing Cx43, where CORM-2 reduced the rate of dye uptake from 48 ± 3 to 25 ± 2 AU/min (Fig. 6C). As negative control we used non-transfected HeLa cells (parental cells); in these, 1 \( \mu \)M CORM-2 had no effect on the rate of dye uptake (6.3 ± 0.7 before versus 6.8 ± 0.4 AU/min after CORM-2) (Fig. 6D). These data demonstrate that the effect of CORM-2 on Cx46 HCs is independent of the cell type and that CORM-2 not only inhibits Cx46 HCs, but also Cx43 HCs. Because regulation of HCs can be altered in overexpressing HeLa cells, we also tested the effect of CORM-2 in cells with native HC expression. In MCF-7 cells, a human breast cancer cell line that expresses Cx43 and Cx46 (21) (Fig. 6E), a decrease of dye uptake by CORM-2 was also observed; uptakes were 30 ± 3 before and 10 ± 1 AU/min after 10 \( \mu \)M CORM-2 (Fig. 6D). The basal dye uptake was inhibited by 200 \( \mu \)M La3+ (85.4% inhibition) and 100 \( \mu \)M Gap27 (65% inhibition), suggesting that the basal rate of uptake is mainly mediated by connexin HCs (Fig. 6F). Also, dye uptake was partially sensitive to 5 \( \mu \)M carbobenoxolone (33% inhibition). Because at this concentration carbobenoxolone inhibits Panx, but not connexin HCs (36), the results suggest that ~1/5 of the dye uptake could be mediated by Panx hemichannels.

CORM-2 Induces Cx46 Carboxylation in Vitro—If the effect of CO on the HCs is direct, it could be mediated by carboxylation of Cx46. Protein carbonyls were estimated from derivatization with 2,4-dinitrophenylhydrazine coupled to a reaction that
forms hydrazine, which is measured by its absorbance at 370 nm. Fig. 7A shows a statistically significant increase in the absorbance after exposure to 100 μM CORM-2 for 3 min at room temperature in both bovine serum albumin (BSA) (from 0.0243 ± 0.0015 to 0.0425 ± 0.0015) and Cx46 (from 0.0119 ± 0.0025 to 0.0275 ± 0.0047). No changes in absorbance were observed when 2,4-dinitrophenylhydrazine was omitted. Co-incubation with CORM-2 and 5 mM DTT did not affect the CORM-2 effect (0.029 ± 0.0017). These results show that Cx46 is carbonylated by CO and that this effect is not directly reversed by DTT. If CO decreases HC activity by carbonylation, it may be possible to determine conformational changes by the effect on Trp fluorescence in response to CORM-2 treatment.

Under control conditions Trp fluorescence emission shows a bell shape (Fig. 7B) with maximum emission of 14.8 ± 0.7 AU at 337 ± 0.7 nm. Exposure to 100 μM CORM-2 for 1 min reduced the maximum fluorescence to 3.40 ± 0.18 AU, with a red shift of the emission maximum to 343 ± 0.4 nm. These results indicate important conformational changes of purified Cx46 in response to CO. Exposure to 5 mM β-ME applied 1 min after CORM-2 did not recover Trp fluorescence. The latter result confirms that reducing agents cannot decarbonylate Cx46 in response to CO. Exposure to 5 mM β-ME applied 1 min after CORM-2 did not recover Trp fluorescence. The latter result confirms that reducing agents cannot decarbonylate Cx46 in response to CO.
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**FIGURE 7.** CORM-2 induces Cx46 carbonylation and conformational changes in vitro. A, purified BSA and Cx46 (both at 1.5 mg/ml) were exposed to CORM-2 for 3 min at room temperature. Then, the proteins were assayed simultaneously exposed to 5 mM DTT and 100 μM CORM-2 (n = 3). B, representative Trp spectra of purified Cx46 under control conditions (solid black line) (n = 4), and after exposure to CORM-2 vehicle ethanol (1:500) (dotted black line) (n = 2), 200 μM CORM-2 (solid gray line) (n = 6), or 5 mM β-ME (dotted gray line) (n = 2). Excitation was at 295 nm and emission was measured between 310 and 420 nm. Data are mean ± S.E.; * and ** denote p < 0.05 and 0.01, respectively.

tributed throughout the protein, and it is not possible to ascribe the structural change to a specific domain.

**DISCUSSION**

We found that CORM-2 causes a large reduction in currents through HCs formed by Cx46 expressed in X. laevis oocytes. We conclude that the effect of CORMs on Cx46 HCs is produced by CO, because CO-depleted CORM-2 solution had no inhibitory effect, the CO scavenger hemoglobin prevented the CORM-2 effect, and other CO releasing molecules (CORM-3 and CORM-A1) have the same inhibitory effect. Interestingly, with the CO-depleted CORM-2 solution we observed an increase in HC activity that suggests that unidentified CORM-2 subproducts activate Cx46 HCs. Consistent with this interpretation; CORM-2 subproducts activate non-selective cation currents in human endothelial cells (37).

It is well known that the signaling effects of CO may involve two mechanisms (i) direct carbonylation of proline, threonine, lysine, and/or arginine residues (16) and (ii) activation of guanylyl cyclase, with elevation of cGMP levels and activation of PKG (18). The lack of effects of guanylyl cyclase/PKG specific inhibitors on the response of Cx46 HCs to CORM-2 strongly suggests that the effect of CO does not involve the guanylyl cyclase/PKG pathways. The Cx46 HC current reduction could also result from internalization and proteasomal degradation of carbonylated Cx46. However, our results do not support this hypothesis because the effect of CO was fully and rapidly reversed by GSHe, β-ME, and DTT and the inhibition of Cx46 HC currents by CO was not prevented by a proteasome inhibitor. Therefore, Cx46 carbonylation seems more likely.

The inhibition of Cx46 HCs by CO was fully reversed by extracellular reducing agents, depended on Cx46 extracellular Cys, and did not involve the regulatory C-terminal domain. We also found that CORM-2 inhibits connexin HCs expressed in human cell lines, indicating that the effect of CO is independent of the experimental system and that CO affect both human and rat Cx46 HCs. However, the loss of effect at 10 μM was unexpected and denotes that the CO effect in mammalian cells could be more complex. We also demonstrated that CORM-2 induces Cx46 carbonylation in vitro, which induce Cx46 conformational changes detected by Trp fluorescence. Thus, this work suggests that CO inhibits Cx46 HCs through carbonylation of extracellular residues and that inhibition is reversible in a Cys-dependent mechanism.

CO may induce secondary Cys-carbonylation in response to lipid peroxidation products, a process that can be sensitive to reducing agents (34). Because the inhibition of Cx46 HCs by CO is reversed by cell-membrane impermeable reducing agents, the possibility of a secondary Cys carbonylation cannot be ruled out. This possibility is supported by the fact that the inhibition by CO was much reduced in HC formed by Cys-less Cx46. As mentioned above, the effect of CORM-2 was reversed by membrane-impermeable reducing agents. This, in combination with our results in vitro, point to a complex enzyme-mediated decarbonylation mechanism. We found that carbonylation of purified Cx46 by CO was not reversed by DTT and that the conformational changes induced by CO were not reverted by β-ME. Thioredoxin- and peroxiredoxin-dependent decarbonylation mechanisms have been proposed (33, 34). Interestingly these two enzymes can act at the extracellular face of the plasma membrane (38), suggesting that this enzymatic mechanism may be involved in our system.

Most of our experiments were done at 100 μM CORM-2. At this concentration and exposure time of usually 3 min, CORM-2 should release about ~75 μmol of CO/liter of solution (39, 40). The physiological concentration of CO is in the range of a few micromolar (41) and we estimated the IC₅₀ at 3.4 μM CORM-2. Therefore, Cx43 and Cx46 HCs can be good sensors of physiological and pathological changes in CO concentration, confirming the regulation of connexin HCs by redox potential (42). Epithelial lens cells in culture express HO-1 in response to an oxidative stress (43). Because lens fibers express Cx46, it is possible that the CO generated in response to cellular stress keeps the HCs closed, preventing cell damage. Because HCs can play important roles in the progression of certain forms of cancer (44), our results may help to understand the molecular mechanism of CO as an anticancer agent (18). The present results suggest the possibility of targeting the CO signaling pathway to treat disorders such as cancer, where activation of Cx46 HCs is involved in the resistance of tumors to hypoxia.

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