Antimicrobial Action of Carbon Monoxide-Releasing Compounds

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Carbon monoxide (CO) is endogenously produced in the human body, mainly from the oxidation of heme catalyzed by heme oxygenase (HO) enzymes. The induction of HO and the consequent increase in CO production play important physiological roles in vasorelaxation and neurotransmission and in the immune system. The exogenous administration of CO gas and CO-releasing molecules (CO-RMs) has been shown to induce vascular effects and to alleviate hypoxia-reoxygenation injury of mammalian cells. In particular, due to its anti-inflammatory, antiapoptotic, and antiproliferative properties, CO inhibits ischemic-reperfusion injury and provides potent cytoprotective effects during organ and cell transplantation. In spite of these findings, the relationship of CO with the biology and pathology of mammals, nothing is known about the action of CO on bacteria. In the present work, we examined the effect of CO on bacterial cell proliferation. Cell growth experiments showed that CO caused the rapid death of the two pathogenic bacteria, Escherichia coli and Staphylococcus aureus, particularly when delivered through organometallic CO-RMs. Of importance is the observation that the effectiveness of the CO-RMs was greater in near-anaerobic environments, as many pathogens are anaerobic organisms and pathogen colonization occurs in environments with low oxygen concentrations. Our results constitute the first evidence that CO can be utilized as an antimicrobial agent. We anticipate our results to be the starting point for the development of novel types of therapeutic drugs designed to combat antibiotic-resistant pathogens, which are widespread and presently a major public health concern.

MATERIALS AND METHODS

Reagents. The different sources or references for CO were as follows: tricarbonylchlorohromium(II) dimer (CORM-2), Sigma; tricarbonyldihalogenorhodium(III) carboxylato) ruthenium(II) (CORM-3), reference 6; bromo(pentacarbonyl)manganese (ALF 021), reference 5; and tetraethylammonium molybdenum pentacarbonyl bromide (ALF 062), reference 2. All compounds were freshly prepared as 10 mM stock solutions by dissolution in dimethyl sulfoxide, pure distilled water, or methanol.

Bacterial strains and growth conditions. E. coli K-12 ATCC 23716 and S. aureus NCTC8325 were grown in minimal salts (MS) medium (1.3% [wt/vol] Na2HPO4, 0.3% [wt/vol] KH2PO4, 0.05% [wt/vol] NaCl, and 0.1% [wt/vol] NH4Cl supplemented with 20 mM glucose, 2 mM MgSO4, 100 μM CaCl2, and 0.25% [wt/vol] Casamino Acids) and in Luria-Bertani (LB) medium (1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, and 1% [wt/vol] NaCl), respectively, under different oxygen supply conditions. Aerobic experiments were undertaken with closed flasks to one-half of their volume, anaerobic conditions were produced in rubber-sealed flasks that, once filled with medium and closed, were extensively fluxed with nitrogen gas.

CO gas and CO-RM treatment. Overnight cultures of E. coli or S. aureus grown in LB or tryptic soy broth, respectively, were used to inoculate fresh MS medium (E. coli) or LB medium (S. aureus), and the cultures on fresh medium were incubated at 37°C under the required aeration conditions to an optical density at 600 nm of 0.3. At this point, cells were exposed to a flux of CO gas for 15 min or to CO-RMs. Untreated cells were bubbled with nitrogen gas or treated with dimethyl sulfoxide, water, or methanol, depending on the solvent used to dissolve the CO-RM.
RESULTS AND DISCUSSION

The effect of CO on the viability of bacteria was investigated first by the direct delivery of CO gas. The administration of CO gas, fluxed into the growing cultures, led to a significant growth impairment of *E. coli* and *S. aureus* (Fig. 1).

To evaluate the potential of CO-RMs, the compounds indicated in Fig. 2 were selected. CORM-2 and CORM-3 are active in a variety of CO-mediated biological processes, both in vitro and in vivo (9). In the first series of experiments, the effect of CO released from CORM-2 on the growth of *E. coli* and *S. aureus* was studied with bacteria cultured under different levels of oxygen supply. Shortly after the exposure to CORM-2, the percentage of surviving cells significantly diminished (Fig. 3). Experiments using water-soluble CORM-3 revealed that, although requiring higher concentrations than CORM-2 due to its...
chemical composition, the compound also strongly decreased the viability of *E. coli* and *S. aureus* cells (Fig. 4). However, while the addition of CORM-3 resulted in a strong inhibition of *E. coli* cell growth, *S. aureus* was more resistant to CORM-3 (Fig. 4A), particularly under aerobic conditions. In general, the action of the two compounds was rapid and extended over time, as cells did not resume growth over the subsequent 4 h (Fig. 3 and 4) or after 8 h (data not shown).

In order to examine whether the bactericidal effect of CO-RMs was due to CO, cell growth experiments with CO-RMs were also performed in the presence of Hb, a high-affinity CO scavenger. In all cases, the bactericidal effect on *E. coli* and *S. aureus* was completely lost (Fig. 3B and 4B), thus demonstrating that the antimicrobial action of CO-RMs is dependent on their release of CO.

Bactericidal activity has been defined as a ratio of the MBC to the MIC of <4 (14). The determination of the CORM-2 MBC/MIC ratios for *E. coli* and *S. aureus* to be 1.5 and 1.0, respectively, revealed the bactericidal character of CORM-2.

The two other CO-RMs used to investigate the bactericidal effect of CO, namely, manganese carbonyl ALF 021 and molybdenum carbonyl ALF 062, were also seen to be capable of strongly reducing the viability of *E. coli* and *S. aureus* (Fig. 5 and 6). Again, the addition of Hb completely eliminated the harmful action of ALF compounds on the two bacteria (Fig. 5 and 6). Furthermore, to ensure that the activity of ALF 062 was not related to its decomposition products, we tested the effects of tetraethyl ammonium bromide, sodium molybdate, and a solution of inactivated ALF 062, obtained after the cessation of CO release (see Materials and Methods), on bacterial growth. None of these compounds had bactericidal properties or altered growth kinetics (data not shown). Therefore,
the bactericidal effects of ALF 062 are due to its capacity to release CO.

It should be mentioned that neither CORM-2 nor CORM-3 releases CO gas when dissolved in the media utilized, even at concentrations higher than those used in our experiments (Table 1). Furthermore, although ALF 021 and ALF 062 release CO gas upon dissolution in the medium, they do so in rather small amounts within the time scale of the experiment (Table 1). However, inductively coupled plasma mass spectrometry analysis of E. coli cells incubated with ALF 062 revealed a very large increase in the content of Mo (155 μg g⁻¹) compared to that in control cells (2.5 μg g⁻¹), confirming that the Mo from ALF 062 accumulates inside the E. coli cells, where it releases CO to the cellular targets.

Since the bactericidal effect of the CO-RMs does not require the release of CO gas to the extracellular medium (Table 1), we must conclude that CO has to be delivered to the cellular targets directly from the CO-RMs. Because Mo from bactericidally active (CO-loaded) ALF 062 is found to accumulate rapidly within cells, we infer that it transports CO and delivers it into the intracellular space, where it reaches the cellular targets and causes the decrease of bacterial cell viability. If Hb is present in the medium, the high affinity of Hb for CO results in a fast transfer (or abstraction) of the active CO from the CO-RMs (or from gas) to the protein hemes and the effective scavenging of CO as CO-Hb (see below). Under these conditions, no CO will be available for intracellular delivery and the cells remain alive.

Albeit with some minor deviations, the general pattern of our results shows that CO-RM toxicity is enhanced when growth occurs under lower oxygen concentrations. For example, ALF 021 was more effective in reducing the viability of E. coli cells grown anaerobically (200 μM ALF 021) than that of cells grown aerobically (500 μM ALF 021). The augmentation of the effect of CO at low oxygen concentrations may be explained by the preferential binding of CO to the ferrous form of heme proteins, which are predominant under reducing environments. More importantly, the bactericidal effect of CO-RMs under anaerobic conditions indicates that growth inhibition is not restricted to the impairment of the respiratory chain by the binding of CO to cytochrome oxidase, which is likely to contribute to the bactericidal activity of these compounds under aerobic conditions. This fact is quite important since pathogen colonization occurs in near-anoxic environments and since many pathogens are anaerobic organisms. On the other hand, the type of bacterial cell wall also seems not to interfere with the action of CO-RMs, as judged by the similar decreases in cell viability observed for the gram-positive (S. aureus) and gram-negative (E. coli) species upon treatment with the same CO-RM. Hence, CO-RMs have the potential for use as bactericides against a wide range of microorganisms independently of the type of bacterial cell wall and oxygen growth requirements.

The difference between the degrees of action of dissolved molecular CO gas and CO-RMs is striking. When administered as gas, CO had to be present in rather high concentrations (ca. 1 mM) to become effective as a bactericide. The ability of CO-RMs to accumulate inside bacterial cells before they release CO makes these compounds highly effective CO donors to bacterial targets, thereby strongly enhancing the bactericidal efficacy of CO. In fact, the CO-RMs used in this study were able to transfer CO to Hb to form CO-Hb, as judged by the shift of the Hb Soret band from 413 to 418 nm (data not shown) and by the results depicted in Fig. 3B, 4B, 5, and 6. Hence, CO-RMs are capable of delivering CO to heme-containing molecules, as had been shown before for the rapid carboxylation of myoglobin by CORM-3 (11). Likewise, the carboxylation of Hb by CORM-2 and CORM-3 occurs within the mixing time, while that by ALF 021 and ALF 062 takes place in less than 15 min. It is well known that the biological effect of CO on mammalian cells is due mainly to its interaction with iron-containing proteins, such as the above-mentioned cytochrome oxidase. In addition to heme proteins and sensors, CO may bind to almost all transition metal-containing proteins, giving rise to structural modifications and alterations of their biological functions. Hence, in bacteria, there are a large number of likely intracellular targets that can account for the toxic effect of CO revealed in this study.

In spite of the increasing expectations for the use of CO in medicine (10, 13, 18), until now, the role of CO as a bactericidal compound had remained unexplored. Nevertheless, in the early 1970s it was reported that the addition of CO to an aerobic culture of E. coli caused a decrease in DNA replication (21). However, as the authors of the study did not observe any effect of CO on cells growing anaerobically on glucose, they concluded that the inhibition of DNA synthesis in cells grown under aerobic conditions was not due to a direct effect on the replication apparatus but resulted from indirect effects, such as ATP or deoxynucleoside triphosphate depletion (21). In more recent years, in spite of several public concerns, CO has been used by the food industry to generate the bright red color of the dark muscle tissue of meat and fish, which results from the great affinity of CO for the Fe(II) binding site of myoglobin. Interestingly, a very recent study of the influence of different packing systems on meat preservation indicated that packages to which CO gas had been added exhibited less bacterial growth than other packages. These results suggest that CO may be one of the packaging gases responsible for the inhibition of the growth of microorganisms (1). We now show that CO and, in particular, CO-RMs have the ability to kill bacteria under aerobic and anaerobic conditions. We submit that CO-RMs constitute a novel class of antibacterial molecules that may become drug candidates upon the development of safe and controllable methods of CO delivery to bacterial targets that avoid the in vivo scavenging of CO by the red blood cells (10). In particular, nonsystemic bactericides may be a relatively easy application for CO-RMs. Although this is a first visual-

### Table 1. CO released into medium by CO-RMs

<table>
<thead>
<tr>
<th>CO-RM (conc, mM)</th>
<th>CO equivalent in MS medium at:</th>
<th>CO equivalent in LB medium at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 min</td>
<td>240 min</td>
</tr>
<tr>
<td>CORM-2 (5)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CORM-3 (12)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ALF 021 (6)</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>ALF 062 (6)</td>
<td>1.4</td>
<td>3.8</td>
</tr>
</tbody>
</table>

* Amounts of CO are expressed as CO equivalents (number of CO groups released per CO-RM molecule).
ization of a still very distant goal, bactericides based upon completely new concepts are urgently required, as the emergence and spread of drug-resistant bacterial pathogens reveal a concerning decrease in the effectiveness of currently available antibiotics.

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REFERENCES


