Antithrombotic Properties of Water-Soluble Carbon Monoxide-Releasing Molecules

Karol Kramkowski, Agnieszka Leszczynska, Andrzej Mogielnicki, Stefan Chlopicki, Andrzej Fedorowicz, Elzbieta Grochal, Brian Mann, Tomasz Brzoska, Tetsumei Urano, Roberto Motterlini, Wlodzimierz Buczko

Objective—We compared the antithrombotic effects in vivo of 2 chemically different carbon monoxide–releasing molecules (CORM-A1 and CORM-3) on arterial and venous thrombus formation and on hemostatic parameters such as platelet activation, coagulation, and fibrinolysis. The hypotensive response to CORMs and their effects on whole blood gas analysis and blood cell count were also examined.

Methods and Results—CORM-A1 (10–30 µmol/kg, i.v.), in a dose-dependent fashion, significantly decreased weight of electrically induced thrombus in rats, whereas CORM-3 inhibited thrombosis only at the highest dose used (30 µmol/kg). CORM-A1 showed a direct and stronger inhibition of platelet aggregation than CORM-3 in healthy rats, both in vitro and in vivo. The antiaggregatory effect of CORM-A1, but not CORM-3, correlated positively with weight of the thrombus. Concentration of active plasminogen activator inhibitor-1 in plasma also decreased in response to CORM-A1, but not to CORM-3. Neither CORM-A1 nor CORM-3 had an effect on plasma concentration of active tissue plasminogen activator. CORM-3, but not CORM-A1, decreased the concentration of fibrinogen, fibrin generation, and prolonged prothrombin time. Similarly, laser-induced venous thrombosis observed intravitally via confocal system in green fluorescent protein mice was significantly decreased by CORMs. Although both CORM-A1 and CORM-3 (30 µmol/kg) decreased platelets accumulation in thrombus, only CORM-A1 (3–30 µmol/kg) inhibited platelet activation to phosphatidylserine on their surface.

Conclusion—CORM-3 and CORM-A1 inhibited thrombosis in vivo, however CORM-A1, which slowly releases carbon monoxide, and displayed a relatively weak hypotensive effect had a more pronounced antithrombotic effect associated with a stronger inhibition of platelet aggregation associated with a decrease in active plasminogen activator inhibitor-1 concentration. In contrast, the fast CO releaser CORM-3 that displayed a more pronounced hypotensive effect inhibited thrombosis primarily through a decrease in fibrin generation, but had no direct influence on platelet aggregation and fibrinolysis. (Arterioscler Thromb Vasc Biol. 2012;32:2149-2157.)

Key Words: carbon monoxide ▪ carbon monoxide–releasing molecules ▪ fibrin generation ▪ green fluorescent protein mice ▪ intravital microscopy ▪ plasminogen activator inhibitor-1 ▪ platelet aggregation ▪ rat ▪ thrombosis

Carbon monoxide (CO) is physiologically present in the human body, and its level is regulated through the enzymatic degradation of heme by heme oxygenase enzymes (HO-1 and HO-2). Despite being renown as the silent killer, experimental data have revealed certain unexpected benefits of small doses of CO gas in the cardiovascular system, primarily associated with its important effects on vascular tone, blood pressure, inflammation, cell proliferation, apoptosis, and cytoprotection against tissue injury. More recent data indicate that CO may also affect thrombosis, a major complication caused by multiple vascular pathologies. Indeed, CO paradoxically increases the formation of fibrin and inhibits fibrinolysis in vitro studies, whereas it exerts antithrombotic effect in vivo, which involves activation of fibrinolysis. Notably, a direct role of CO gas in mitigating the process of thrombosis was assessed by True et al. These authors found that Hmox-1 knockout mice display a prothrombotic phenotype characterized by damage of endothelial cells and apoptosis, platelet activation, elevation in both tissue factor and von Willebrand factor, and reactive oxygen species. In addition, transplantation of HO-1-deficient bone marrow–derived progenitor cells into wild-type HO-1
animals resulted in rapid arterial thrombosis. Interestingly, all these pathological phenotypes were rescued by administration of CO gas. In another study by Chen et al., it was shown that in carotid arteries injury model of apolipoprotein E-deficient mice a significant antithrombotic effect can be achieved by exposition to CO inhalation (250 ppm) for 2 hours or by treatment with an adenovirus bearing the HO-1 gene.

Because Motterlini et al. described CO-releasing molecules (CORMs) as a novel approach to deliver precise amounts of CO within biological systems, several reports confirming the pharmacological efficacy of CO have appeared in the literature. The first class of CORMs studied were composed of transition metal carbonyls, complexes of metals surrounded by CO groups as coordinated ligands. CO-releasing molecule-1 (manganese decacarbonyl; $t_{1/2} \approx 1$ minute) and CORM-2 (tricarbonyldichlororuthenium(II) dimmer; $t_{1/2} \approx 1$ minute), soluble in ethanol and dimethyl sulfoxide and release CO either by photodissociation or by ligand substitution. These carbonyl complexes have been shown to exert important pharmacological effects both in vitro and in vivo (Material I in the online-only Data Supplement). In the context of thrombosis, Chen et al. demonstrated that CORM-2, but not an inactive counterpart that does not release CO, rescues Hmox-1 knockout mice against the development of arterial thrombosis after allo-genic aortic transplantation. Interestingly, this antithrombotic action of CORM-2 was associated with a markedly reduced platelet aggregation within the graft. However, it has been shown that CO liberated from CORM-2 in vitro significantly enhances fibrinogen (Fg)–dependent coagulation kinetics in a concentration-dependent manner in plasma. Advances in the development of novel CORMs led to the synthesis and characterization of water-soluble compounds, the best representation of which is CORM-3 (tricarbonylchloro(glycinato)ruthenium(II); $t_{1/2} = 1$ minute). In vitro and in vivo experiments demonstrated that CO liberated from CORM-3 improves endothelial function, mediates anti-inflammatory responses, protects against ischemia-reperfusion injury, and prevents organ rejection after transplantation. Antiaggregatory activities of CORM-3 have also been demonstrated in human platelets in vitro. Interestingly, another class of water-soluble CORMs that do not contain metals (boranocarbonates) has been described as pharmacologically active.

CORM-A1, the best characterized of this class, releases CO in vivo at a much slower rate than CORM-3 and the kinetic of CO release is strictly dependent on pH and temperature ($t_{1/2} = 21$ minutes at pH = 7.4 and 37°C). Intuitively, improved pharmacokinetic profiles would make this particular spontaneous CO releaser an ideal tool to study the physiological properties of CO gas in living organism without the interference of a transition metal carrier.

The aim of the present work was therefore to compare the antithrombotic effects of CORM-A1 and CORM-3 by specifically assessing the effects of these 2 CORMs on hemostatic parameters such as platelet aggregation, coagulation, and fibrinolysis, as well as on blood cell count and blood gas analysis.

Materials and Methods

Animals

Male Wistar rats (120–150 g) were purchased from the Center of Experimental Medicine of Medical University of Białystok (Poland) and green fluorescent protein (GFP)–expressing transgenic mice, based on the C57BL/6J strain (GFP mice), were supplied by Dr Okabe (Osaka University, Osaka, Japan) and maintained in Animal Facility of Hamamatsu University School of Medicine (Hamamatsu, Japan) according to Good Laboratory Practice rules. Animals were housed in a room with a 12 hour light/dark cycle, grouped in cages as appropriate and allowed to have access to tap water and a standard rat/mice chow. All the procedures involving animals were approved by a bioethical committee and conducted in accordance with the institutional guidelines that are in compliance with national and international laws and Guidelines for the Care and the Use of Animals in Biomedical Research.

Chemicals and Drugs

CORM-3 and CORM-A1 were synthesized as previously described. Inactive CORM-3 (iCORM-3) was prepared from CORM-3 by leaving the solution of CORM-3 in phosphate buffer solution for 48 hours followed by bubbling with N₂. iCORM-A1 was prepared by slight acidification of the solution with HCl, then rebubbling and bubbling with N₂. Pentobarbital (Vetbutal, Biowet, Pulawy, Poland; or Nembutal, Dainippon Pharmaceutical, Osaka, Japan), PBS (Biomed, Lublin, Poland), Tris buffer (Sigma-Aldrich, Hamburg, Germany), collagen (Chrono-Log Corp, Havertown, PA), Alexa Fluor 568 dye (Invitrogen, Carlsbad, CA; Molecular Probes, Eugene, OR), Annexin 5A (KOWA Pharmaceuticals, Tokyo, Japan), and ready-to-use kits for blood cell count and gas blood analysis were used in the study. Routine laboratory reagents to determine prothrombin time, activated partial thromboplastin time, and Fg levels in vitro in rat plasma were purchased from HemosIL, Instrumentation Laboratory (Lexington, MA). Rat plasminogen activator inhibitor-1 (PAI-1) and tissue plasminogen activator ELISA kits were purchased from Hyphen BioMed, Cergy, France.

Administration of CORMs: Experimental Protocol in Rats

CORM-3 or CORM-A1 were administered into the right femoral vein at doses of 3 (n=5), 10 (n=17), 30 (n=12) µmol/kg, or 3 (n=10), 10 (n=11), 30 (n=11) rats µmol/kg, respectively. CORMs were injected 15 minutes before the induction of carotid artery injury. Doses of CORMs were chosen on the basis of previously published data. iCORMs (iCORM-3 or iCORM-A1, 30 µmol/kg; n=6) were used as negative controls. Vehicle (VEH; 0.9% NaCl; n=32) served as a control to CORMs-treated rats and the PBS (n=5) served as a control to iCORMs-treated animals.

Administration of CORMs: Experimental Protocol in Mice

CORM-3 or CORM-A1 were administered into the right femoral vein at doses of 3 (n=4), 30 (n=7) µmol/kg, or 3 (n=5), 30 (n=9) µmol/kg, respectively. CORMs were injected 15 minutes before the mesenteric vein injury. Doses of CORMs were chosen on the basis of previously published data. iCORMs (iCORM-3 or iCORM-A1, 30 µmol/kg; n=3) were used as negative controls. VEH (0.9% NaCl; n=9) served as a control to CORMs-treated rats and the PBS (n=3) served as a control to iCORMs-treated animals.

Measurement of Platelet Aggregation in Rats

Collagen-stimulated platelet aggregation in vitro and ex vivo in a citrated whole blood was evaluated with the impedance method as described previously and measured in a Whole Blood Lumi-Aggregometer (Chrono-Log Corp).
For in vitro experiments, blood samples were drawn from normal rats into 3.13% trisodium citrate in a volume ratio 10:1. After 13 minutes of incubation at 37°C with 0.9% NaCl (volume ratio 1:1), samples were mixed with CORM-A1 (final concentrations: 1 mmol/L, 0.5 mmol/L, and 0.1 mmol/L) or CORM-3 (final concentrations: 5 mmol/L, 1 mmol/L, and 0.5 mmol/L) or VEH (0.9% NaCl). Collagen (5 µg/mL) was added 2 minutes after.

For ex vivo experiments, blood samples were collected from rats with electrically stimulated thrombosis 75 minutes after administration of VEH (0.9% NaCl) or CORMs in doses of 3, 10, 30 µmol/kg or iCORMs in doses of 30 µmol/kg into 3.13% trisodium citrate in a volume ratio of 10:1 (thrombotic experiment). After 15 minutes of incubation at 37°C with 0.9% NaCl (volume ratio 1:1), collagen (5 µg/mL) was added.

Blood samples were collected from normal rats 15 minutes after CORM-A1 or CORM-3 administered in doses of 10 µmol/kg and 30 µmol/kg, and 60 minutes after CORM-A1 or CORM-3 administered in doses of 10 µmol/kg. iCORMs were administered in doses of 30 µmol/kg 15 or 60 minutes before blood collection. After 15 minutes of incubation at 37°C with 0.9% NaCl (volume ratio 1:1), collagen (5 µg/mL) was added.

In in vitro and ex vivo experiments, changes in resistance were registered during 6 minutes. The maximal extension of the aggregation curve at the sixth minute was expressed as a percentage of control response.

### Induction of Arterial Thrombosis in Rats

Rats were anesthetized with pentobarbital (40 mg/kg, i.p.) and placed in a supine position on a heated (37°C) operating table. The left femoral vein was cannulated to administer the drug. Arterial thrombosis was induced by electric stimulation of the right common carotid artery as previously described. Briefly, the anode, a stainless steel L-shaped wire, was inserted under the artery and connected with a cannula filled with heparin solution (150 IU/mL), placed in the left common carotid artery and connected to a pressure transducer (Plugsys; Transonics System, Ithaca, NY), as described previously.

### Blood Pressure Measurements

Rats were anesthetized with pentobarbital (40 mg/kg, i.p.) and placed in a supine position on a heated (37°C) operating table. The mean blood pressure was measured in anesthetized rats directly through a cannula filled with heparin solution (150 IU/mL), placed in the left common carotid artery and connected to a pressure transducer (Plugsys; Transonics System, Ithaca, NY), as described previously.

### Coagulation Parameters and Fibrin Generation

Prothrombin time, activated partial thromboplastin time, and Fg levels (Clauss method) were determined according to the kit’s manufacturer’s instructions using Coag-Chrom 3003 apparatus (Bio-kse1, Grudziadz, Poland). A more sensitive assay for fibrin generation was used on the basis of a method described previously and modified by us. Fibrin generation curves were created by recalcification of rats’ plasma samples directly in microplate wells with CaCl2 (36 mmol/L) dissolved in Tris buffer (66-mmol/L Tris; 130-mmol/L NaCl; pH=7.4) in 37°C. Optical density increases in the wells (as a result of fibrin generation) were measured via the microplate reader (Dyneex Technologies, Chantilly, VA) in 1-minute intervals for 14 minutes and expressed as an area under the curve. Time-points analysis was calculated using the values of percentage of basal optical density.

### Fibrinolysis Parameters

Concentrations of total PAI-1 antigen, active PAI-1, and tissue plasminogen activator were analyzed using ELISA techniques using a microplate reader (Dyneex Technologies) according to the kit’s manufacturer’s instructions.

### Blood Cell Count

Blood cell count was assessed by an animal blood counter (ABC Vet, Darmstadt, Poland) collected from the heart of the rat after thrombus removal.

### Blood Gas Analysis

In anesthetized Wistar rats (body weight=160–170 g) trachea was cannulated and lungs were artificially ventilated (7025 Rodent Ventilator, Ugo Basile) with a mixture of oxygen and isoflurane (1.1%–1.2%) with positive pressures (rate of 80 breaths per minute; tidal volume=2.4–2.6 mL). Carotid artery was cannulated. CORMs (CORM-A1, CORM-3, iCORM-A1, and iCORM-3) were dissolved in saline. 15 seconds before giving to the dorsal penile vein (dose 30 µmol/kg) and arterial blood samples were collected from the carotid artery before 10 minutes, and 65 minutes after CORMs injection. Saline treated rats served as a control group. Blood gasometry parameters were measured by pHox analyzer (Nova Biomedical, Waltham, MA) immediately after collection of probes.

### Statistical Analysis

The data are shown as means±SEM and analyzed using either a Mann-Whitney test (when normality test failed) or an unpaired t test.
Table. Coagulation and Blood Cell Count Parameters (Plasma) Measured in Rats Treated with 0.9% NaCl (VEH) or CORM-A1 (3, 10, 30 µmol/kg) or CORM-3 (3, 10, 30 µmol/kg) or iCORMs (30 µmol/kg)

<table>
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<th>VEH 30 µmol/kg</th>
<th>iCORM-A1 3 µmol/kg</th>
<th>CORM-A1, µmol/kg</th>
<th>iCORM-A1, 10 µmol/kg</th>
<th>CORM-A1, 30 µmol/kg</th>
<th>iCORM-3, 30 µmol/kg</th>
<th>CORM-3, µmol/kg</th>
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<tr>
<td><strong>PT, s</strong></td>
<td>21.10±0.47</td>
<td>24.44±1.06</td>
<td>18.84±0.39</td>
<td>20.72±0.42</td>
<td>20.35±0.74</td>
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<td><strong>APTT, s</strong></td>
<td>24.26±1.02</td>
<td>18.84±0.63</td>
<td>26.09±1.19</td>
<td>21.49±1.43</td>
<td>21.60±0.79</td>
<td>19.77±0.60</td>
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<td><strong>PAI-1 total, ng/mL</strong></td>
<td>0.42±0.02</td>
<td>0.34±0.02</td>
<td>—</td>
<td>—</td>
<td>0.38±0.03</td>
<td>0.31±0.03</td>
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<tr>
<td><strong>PAI-1 activity, ng/mL</strong></td>
<td>1.65±0.09</td>
<td>1.43±0.1</td>
<td>1.78±0.25</td>
<td>1.77±0.20</td>
<td><strong>1.28±0.08</strong></td>
<td>1.64±0.17</td>
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<td><strong>tPA, ng/mL</strong></td>
<td>1.83±0.1</td>
<td>1.61±0.2</td>
<td>—</td>
<td>—</td>
<td>1.59±0.04</td>
<td>1.71±0.55</td>
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**Blood Cell Count**

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<tr>
<td><strong>WBC, 10⁶/µL</strong></td>
<td>1.70±0.07</td>
<td>1.7±1.16</td>
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<td>1.71±0.18</td>
<td>1.67±0.14</td>
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<td><strong>RBC, 10⁹/µL</strong></td>
<td>5.91±0.15</td>
<td>5.42±0.07</td>
<td>6.70±0.10</td>
<td>6.39±0.29</td>
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<td><strong>HGB, g/dL</strong></td>
<td>12.28±0.18</td>
<td>11.53±0.07</td>
<td>12.97±0.16</td>
<td>12.67±0.53</td>
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<td>12.45±0.60</td>
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<td><strong>HCT, %</strong></td>
<td>36.16±0.67</td>
<td>32.98±3.45</td>
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<td><strong>MCV, fL</strong></td>
<td>59.28±1.17</td>
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<td><strong>MCH, pg per cell</strong></td>
<td>20.37±0.39</td>
<td>22.16±0.26</td>
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<td>19.92±0.30</td>
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<td><strong>MCHC, g/dL</strong></td>
<td>33.18±0.18</td>
<td>36.78±0.28</td>
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<td>33.21±0.27</td>
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<td><strong>PLT, 10⁵/µL</strong></td>
<td>576.85±11.24</td>
<td>545.80±11.05</td>
<td>603.57±19.19</td>
<td>563.86±25.17</td>
<td>604.29±23.20</td>
<td>634.50±24.48</td>
<td>576.00±11.00</td>
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</table>

VEH indicates vehicle; CORM, carbon monoxide–releasing molecules; iCORM, inactive CORM; PT, prothrombin time; APTT, activated partial thromboplastin time; PAI-1, plasminogen activator inhibitor; tPA, tissue plasminogen activator; WBC, white blood cells; RBC, red blood cells; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, MCH concentration; PLT, platelets.

Data are expressed as mean±SEM.

Bold values indicate \( P<0.05 \) vs VEH.

\*P<0.05 vs VEH.

\**P<0.01.\**
Results

Effect of CORMs and Its Inactive Counterparts on Platelet Aggregation in Normal Rats
CORM-3 (0.5–5 mmol/L) and CORM-A1 (0.1–1 mmol/L) exerted a concentration-dependent antiaggregatory effect on rat’s platelets in vitro in the whole blood (Figure 1). However, CORM-A1 was much more potent than CORM-3 and at a concentration of 1 mmol/L completely blocked collagen-induced platelet aggregation. In contrast, CORM-3 reached its maximal effect also at 1 mmol/L, but it inhibited platelets aggregation only by 50%. iCORMs used at the same concentrations as CORMs did not influence platelet aggregation in the same experimental set-up (Figure 1).

In ex vivo experiments, the effect of CORM-A1 (30 µmol/kg) on platelet aggregation was more pronounced as compared with CORM-3 when administered into healthy animals 15 minutes before blood collection (Figure 2A). Similarly, when CORMs (10 µmol/kg) were administered 60 minutes before blood collection, the antiplatelet effect of CORM-A1 measured at 60 minutes versus 15 minutes after administration was significantly more pronounced as compared with CORM-3 (Figure 2B). iCORMs did not influence platelet aggregation ex vivo in healthy rats both at 15 and 60 minutes after administration (Figure 2B).

Effect of CORMs and Its Inactive Counterparts on Blood Gas Analysis in Normal Rats
In the group of rats treated with CORM-A1 (30 µmol/kg), blood carboxyhemoglobin (COHb) concentration increased significantly 10 minutes after administration from 2.5±0.66% (baseline) to 8.9±2.1% (P ≤0.01), but dropped to baseline values 65 minutes after CORM-A1 administration (2.9±0.5% for control group; nonsignificant). No changes in COHb concentration were observed in animals treated with CORM-3 or both iCORMs, as compared with control group. Other blood parameters did not change after CORMs administration (Material III in the online-only Data Supplement).

Effect of CORMs and Its Inactive Counterparts on Platelet Aggregation in Thrombotic Rats
CORM-A1 administered at doses of 10 and 30 µmol/kg inhibited platelet aggregation in whole blood collected immediately after thrombus removal (74.7±20% and 57.1±10% of VEH, respectively). In contrast, the effect of CORM-3 was less pronounced showing only a trend of inhibition on platelet aggregation at the highest dose used (30 µmol/kg; Figure 3A). iCORM-3 and iCORM-A1 failed to inhibit platelet aggregation (Figure 3A).

Effect of CORMs and Its Inactive Counterparts on Electrically Induced Arterial Thrombus Formation
CORMs inhibited arterial thrombus formation. In the case of CORM-A1, doses of 10 and 30 µmol/kg decreased thrombus weight from 0.81±0.02 mg to 0.69±0.07 mg (P <0.05) and 0.62±0.03 mg (P<0.001), respectively. CORM-3 was only effective at a dose of 30 µmol/kg decreasing thrombus weight to 0.7±0.04 mg (Figure 3B). iCORM-3 and iCORM-A1 failed to influence thrombus weight (Figure 3B). It is interesting to note that the inhibition of platelet aggregation correlated with the decrease in thrombus weight only in the case of CORM-A1 (r=0.60; P<0.01; Material IVA in the online-only Data Supplement), but not in the case of CORM-3 (Material IVB in the online-only Data Supplement).

Effect of CORMs and Its Inactive Counterparts on Laser-Induced Venous Thrombosis Observed Intravitally in Mice
CORMs inhibited venous thrombus formation. In the case of CORM-A1, only a dose of 30 µmol/kg decreased maximal fluorescence intensity of GFP from 4.70±0.23 units to 1.97±0.23 units (P<0.01), while fluorescence intensity of ANX was decreased by doses of 3 and 30 µmol/kg from 1.97±0.23 units to 0.96±0.15 units and 0.59±0.17 units (P<0.05 and P<0.01, respectively; Figure 4A and 4B). CORM-3 was significantly effective only at a dose of 30 µmol/kg decreasing fluorescence intensity of GFP from 4.70±0.58 units to 1.92±0.57 units (P<0.05). Moreover, CORM-A1 decreased ANX fluorescence slightly stronger than CORM-3 (P=0.06; Figure 4B). iCORMs were without any effect on GFP or ANX fluorescence (data not shown).

Effect of CORMs on Blood Pressure
CORM-3 given at a dose of 30 µmol/kg exerted significant hypotensive effect (P≤0.05) starting =10 minutes after the
injection, which increased ($P \leq 0.01$) =30 minutes after the injection. CORM-A1 in the same dose also exerted hypotensive response ($P \leq 0.05$), but this response appeared later as compared with the effect of CORM-3 (=20 minutes after the injection) and was less pronounced. CORMs used at the lower doses did not change mean blood pressure compared with control values. Also iCORMs did not exert hypotensive effects (Material VA and VB in the online-only Data Supplement).

Effect of CORMs and iCORMs on Coagulation Parameters in Thrombotic Rats
CORM-3 significantly reduced the concentration of plasma Fg in vivo at the dose of 30 $\mu$mol/kg (1.88±0.06 ng/mL) compared with VEH-treated animals (2.21±0.13 ng/mL; $P<0.01$), while CORM-A1 was without effect (Figure 5). iCORMs failed to affect the concentration of plasma Fg in vivo (2.52±0.1 ng/mL with iCORM-A1 and 2.29±0.10 ng/mL with iCORM-3 versus 2.32±0.11 mg/mL with PBS). Additionally, fibrin generation was significantly inhibited by CORM-3. In fact, fibrin generation decreased from 0.919±0.04 units in the VEH-treated group to 0.485±0.05 and 0.194±0.14 units in animals treated with 10 and 30 $\mu$mol/kg CORM-3, respectively ($P<0.01$; Figure 5). Consequently, prothrombin time was prolonged in rats’ plasma treated with 30 $\mu$mol/kg CORM-3 ($P<0.05$; Table), whereas activated partial thromboplastin time did not change in any group. iCORM-A1 (30 $\mu$mol/kg) failed to influence coagulation parameters, whereas 30 $\mu$mol/kg iCORM-3 reduced fibrin generation from 0.998±0.069 (PBS-treated group) to 0.722±0.08 units ($P<0.05$; Figure 5). iCORM-3 significantly decreased fibrin formation in all examined time points of the clotting curve (Material VI in the online-only Data Supplement).

Effect of CORMs and Its Inactive Counterparts on Fibrinolysis Parameters in Thrombotic Animals
The concentration of active PAI-1 was significantly decreased in plasma of rats treated with 30 $\mu$mol/kg CORM-A1 when compared with the VEH-treated ($P<0.05$), but CORM-3 was without effect at any doses tested (Table). The concentrations of total PAI-1 antigen in rats treated with 30-$\mu$mol/kg CORM-A1 or CORM-3 did not significantly change when compared with VEH-treated rats. Similarly, the concentrations of active tissue plasminogen activator did not change in rats treated with 30-$\mu$mol/kg CORM-A1 or CORM-3 when compared with VEH-treated group. iCORMs failed to influence fibrinolysis (Table).

Effect of CORMs and Its Inactive Counterparts on Blood Cell Count in Thrombotic Animals
No changes were observed on blood cell count in animals treated with CORMs as well as with iCORMs (Table).

Figure 3. A, Collagen-stimulated platelet aggregation in whole blood from thrombotic rats. B, Thrombus weight in rats treated with 0.9% NaCl (VEH for CORMs) or CORMs; 3, 10, 30 $\mu$mol/kg) or with PBS (VEH for iCORMs) or with iCORMs (30 $\mu$mol/kg) 10 minutes before induction of arterial thrombosis. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs VEH. Data are mean±SEM. VEH indicates vehicle; CORMs, carbon monoxide–releasing molecules; iCORMs, inactive CORMs.

Figure 4. Relative fluorescence intensities of green fluorescent protein (GFP; A) and annexin V (B) in mice treated with 0.9% NaCl (vehicle [VEH]) or carbon monoxide–releasing molecules (CORMs; 30 $\mu$mol/kg) 15 minutes before laser injury of vascular endothelial cells on mesenteric vein *$P<0.05$; **$P<0.01$ vs VEH; ^$P=0.06$ vs CORM-3.
Figure 5. Fibrinogen levels (ng/mL; white columns) and fibrin generation (area under the curve [AUC]; black columns) in plasma of rats developing arterial thrombosis treated with carbon monoxide–releasing molecules (CORMs) 10 minutes before induction of arterial thrombosis. *P<0.05, **P<0.01, ***P<0.001 vs VEH. Data are expressed as mean±SEM. VEH indicates vehicle; iCORM, inactive CORMs.

Discussion

Previous studies performed on smokers or subjects who inhaled CO gas revealed that CO may influence platelet function, coagulation, and fibrinolysis in humans. Recent studies using CORM-2 and CORM-3 provided evidence that non-toxic quantities of CO may also exert antithrombotic effects and inhibition of platelet aggregation.

The data presented here also show for the first time that not only CORM-3, but also CORM-A1 inhibits platelet aggregation in vivo. Because we found that the effect of CORMs was more pronounced at a later time after administration (60 minutes), we wanted to explore whether CORMs may also influence the progression of arterial thrombosis. We were also interested to assess whether the diverse CO-releasing profile of CORM-A1 compared with CORM-3 would differentially influence the activity in vivo.

Electrically induced thrombosis is a valuable model for studying the antithrombotic efficacy of novel compounds because it mimics clinical thrombosis. The results of the present study demonstrate that both CORM-3 and CORM-A1 administered intravenously at micromolar doses inhibited arterial thrombus formation in vivo. Another important finding in our study is that the effect of CORM-A1 is strictly dose dependent and much more potent compared with CORM-3. Interestingly, a significant decrease in thrombus weight correlated with the inhibition of platelet aggregation only in the group treated with CORM-A1 which is known to release CO in a constant and gradual manner over time. The differential effects obtained with these 2 CO releasers are in line with our recent observations. It is not surprising because the intrinsic chemical nature of these 2 compounds and especially time course of CO release differs, as presented in Material I in the online-only Data Supplement. As we previously showed, the direct vasorelaxant effect on the vessels and hypotensive effect (confirmed in the present study) of both CORMs have different nature. Notably, inhibition of NO synthase activity by NG-nitro-L-arginine methyl ester or removal of the endothelium, which significantly inhibited CORM-3–mediated vessel relaxation, failed to prevent the pharmacological effects of CORM-A1, suggesting that the action of CO exerted through the reactivity of boronocarbonate is independent of the endothelial function. Moreover, the blockade of potassium channels with glibenclamide, which is also known to partially attenuate the vasorelaxation by CORM-3, did not have any effect on the vasoactivity elicited by CORM-A1. We know that CORM-A1, unlike CORM-3, possesses some reducing capacities in addition to its ability to liberate CO, and thus may differently affect the response of the vessels. It seems the effects of CORMs, especially CORM-A1 on the vascular hemostasis may be independent on the hemodynamic activity, because at the doses used in the present study it exerted antithrombotic effect in doses that had no influence on blood pressure.

It is also evident that the effects on the thrombosis and platelet of CORM-A1 were more potent when compared with CORM-3. We know that the CORM-A1 releases CO for 1 hour, thus for the whole time of the arterial thrombus development and the platelet aggregation in vivo. In contrast, CORM-3 liberates CO in the first few minutes, thus in the time of platelet activation after vascular injury CO is not present anymore in the circulation. We may assume that high amounts of CO released from CORM-3 in a short time, but not from CORM-A1, are able to inhibit coagulation factors that are presented in the blood before thrombosis induction. It is interesting to note that severe and prolonged bleeding and a higher incidence of hemorrhagic strokes were reported after intoxication with the inhaled CO.

This hypothesis is also supported by our results on the coagulation parameters. The fibrin generation inhibited by CORM-3 strongly correlated with the decrease of Fg levels in plasma. The kinetics of fibrin generation process was also significantly inhibited by CORM-3, suggesting a strong anticoagulant effect. In addition, prothrombin time was also prolonged at the highest dose of CORM-3. These results indicate that the antithrombotic effects of CORM-3 may be only related to the inhibition of the coagulation cascade. The fact that CORM-A1 reduced fibrin generation suggests that this effect might depend on the presence of the ruthenium metal since no changes on this parameter have been observed after treatment of animals with iCORM-A1. However, it cannot be excluded a priori that the effect of CORM-3 in vivo is due to the residual CO groups bound to and released from the metal because only 1 CO per mole is rapidly detected in vitro.

We also found differences in the effect of CORMs on the fibrinolytic parameters. The concentration of active PAI-1 was significantly decreased in plasma of rats treated with CORM-A1 (30 µmol/kg), but not with CORM-3. This discrepancy might also rely on the time course of CO release. During the development of thrombosis, the fibrinolysis process starts after platelets activation and formation of active coagulation plasma factors. Notably, the activated platelets are the main source of active PAI-1 regulating production of plasmin, which limits the growth of thrombus only to the damaged vascular wall. At the time of PAI-1 release from platelets (13–20 minutes after CORM iv administration) only CORM-A1 was still liberating CO, which in turn could interfere with the activity of PAI-1. This is also confirmed by previous studies by Soni et al. Those authors showed that CORM-3 decreased plasma PAI-1 levels, but in their model CORM-3 was administered...
during a 10-minute infusion at 3 mg/kg (which is equivalent to 100 μmol/kg of CORM-3), thus the CO, which was liberated, could have been in contact with the released from platelets PAI-1. However, Nielsen et al. have shown that CORM-2 inhibits fibrinolysis. Nevertheless, their experiments were preformed only in vitro using this lipid-soluble and fast metal-containing CO releaser, which makes it difficult to compare with our results obtained with water-soluble CORMs in vivo. Moreover, all our and others, previous animal studies provide evidence for the profibrinolytic/antithrombotic effects of CO partially due to the suppression of antifibrinolytic molecules, such as PAI-1, and our study for the first time in vivo shows a similar effect of CORM-A1.

To confirm the antithrombotic activity of CORMs we have performed intravital laser-induced venous thrombosis in GFP mice, a novel method for real-time evaluation of the progression of thrombosis. Simultaneously relative fluorescence intensities of GFP and ANX are measured. The GFP fluorescence corresponds to platelet accumulation in area of laser-injured endothelium. ANX only binds platelets that are irreversibly activated and expose platelet phospholipids on their surface. Similarly to reducing thrombus weight in rats, both CORMs decreased fluorescence intensity of GFP in laser-induced venous thrombosis in mice. However, only CORM-A1 inhibited platelet phospholipids exposure on platelet surface (decrease in ANX fluorescence), showing strong antiplatelet effect. Thus, these data further confirm the antithrombotic effect of both CORMs and the antiplatelet effect of CORM-A1.

In the present study, we found that in vitro application of CORM-3 and CORM-A1, respectively, a fast and slow CO releaser, inhibits platelet aggregation in healthy rats in the same manner as previously reported in human platelets by Chlopicki et al. Interestingly, CORM-A1 appears to be a stronger inhibitor of rat platelet aggregation in comparison with CORM-3. Taking into account the pharmacokinetic properties of these 2 water-soluble CORMs, when the antiaggregatory effect is measured after 6 minutes, CORM-3 has completely released CO, whereas CO from CORM-A1 is still slowly and consistently liberated. This difference in the rate of CO release may partially explain why CORM-A1 is more effective, because CO is still being liberated when platelets are activated, thus inhibiting platelet aggregation more efficiently. These data are in line with our recent observation on human platelets showing that in in vitro conditions CORM-A1 is indeed more potent in inhibiting platelet aggregation. These new data point out that CORM-A1 display antiplatelet activities that should be exploited further for its therapeutic use as an antithrombotic drug in vivo.

In the present study, it was important to verify that CO released from CORMs did not affect COHb levels, to ascertain that the doses used did not compromise the oxygen carrying capacity of blood. In blood collected from thrombotic animals, no changes in blood gas parameters such as HCO₃⁻, partial pressure of CO, partial pressure of oxygen, and pH as well as blood COHb levels were observed after treatment with CORM-3. COHb levels increased slightly after CORM-A1 administration but did not reach toxic values. CORMs did not affect blood cell count in any group tested, further excluding a potential toxic effect of CO delivered by these compounds. In conclusion, we report that CORM-A1 possesses antithrombotic activity in vivo in 2 models of thrombosis. CORM-A1, by releasing CO at a slower rate and at not toxic concentrations, also promotes a strong antiplatelet and fibrinolytic activity, without a significant hypotensive effect. In contrast, CORM-3 that releases CO more rapidly is a weaker inhibitor of thrombosis and acts primarily by inhibition of plasma clotting factors and fibrin generation. For the first time we have compared the effect of CO delivered to living organisms on hemostasis using 2 carriers that release different amounts of CO with different times and rates. If the presence of CO in the circulation is prolonged, this gas may modulate platelets function and fibrinolytic activity. In contrast, sufficiently high concentrations of CO should be reached to significantly inhibit fibrin generation. Importantly, our study indicates that it is possible to achieve a significant antithrombotic and antiaggregatory effect with CO-releasing compounds without major hypotensive effects with CORM-A1-like compounds that release CO at a slow rate. Our findings suggest that CORMs provide a novel tool for the design of therapeutic strategies against thrombosisis in cardiovascular diseases such as myocardial infarction and stroke, but further studies are required to validate its feasibility in humans.

Acknowledgments
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Disclosures
None.

References
R. Carbon monoxide released by CORM-3 inhibits human platelets by a fusion reduces infarct size in vivo. 


Antithrombotic Properties of Water-Soluble Carbon Monoxide-Releasing Molecules
Karol Kramkowski, Agnieszka Leszczynska, Andrzej Mogielnicki, Stefan Chłopicki, Andrzej Fedorowicz, Elżbieta Grochal, Brian Mann, Tomasz Brzoska, Tetsumei Urano, Roberto Motterlini and Włodzimierz Buczko

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### SUPPLEMENT MATERIALS

for Kramkowski K: Antithrombotic properties of CO-RMs

**Supplement material I.** Chemical properties of CO-RMs.

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Supplement material II.

Relative changes in the fluorescence intensity of GFP and ANX in the horizontal (X–Y) images.

PS exposure on the platelet surface initiates at a site of laser injury and spatially develops with time during thrombus formation. From focal plane images along the z axis from the vessel wall to the luminal surface of a thrombus taken every 5 s for at least 6 min., the largest horizontal plane was chosen for calculation. The peak intensity of GFP occurred around 50 s after laser injury and then decreased to baseline at 400 s whereas the intensity of ANX gradually increased and reached a plateau at 200 s, which corresponds to the changes of thrombus size and the development of surface-exposed PS, respectively.
**Supplement material III.** Effect of CO-RMs and its inactive counterparts on blood gas analysis in healthy animals 10 and 65 min after administration.

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Data are expressed as mean.
Supplement material IV. A: Correlation between platelet aggregation and thrombus weight in rats treated with CORM-A1 (3, 10, 30 µmol/kg). [Thrombus weight (mg) = 0.46939 + 0.00336 * Platelet aggregation (% of VEH), correlation: r = 0.60227; p < 0.01]. B: Correlation between platelet aggregation and thrombus weight in rats treated with CORM-3 (3, 10, 30 µmol/kg) [Thrombus weight (mg) = 0.75061 + 0.54E-3 * Platelet aggregation (% of VEH), correlation: r = 0.05673; p = ns.]
Supplement material V. Blood pressure measured directly in carotid artery after iv administration of CO-RMs. Data are mean±SEM.
**Supplement material VI.** Effect of CORM-A1 and CORM-3 and its inactive counterparts on fibrin generation in plasma of rats developing arterial thrombosis; *p<0.05, **p<0.01, ***p<0.001 vs VEH. Data are expressed as mean±SEM.