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Investigation into the mechanism(s) of antithrombotic effects of carbon monoxide releasing molecule-3 (CORM-3)

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ABSTRACT

Carbon monoxide (CO) like nitric oxide (NO) has been recognized as activator of soluble guanylate cyclase (sGC) in many physiological functions. Studies, which demonstrate the mechanisms by which CO inhibits platelet aggregation in *in vivo* models, are few. Here we investigated the possible involvement of sGC, NO, plasminogen activator inhibitor (PAI-1) and p38 MAP Kinase in antithrombotic effects of CO released by a novel, water-soluble, CO releasing molecule-3 (CORM-3) using rat. The effects of CORM-3 on *in vitro* and *ex vivo* platelet aggregation induced by thrombin as well as in *in vivo* thrombosis models were studied. When added to rat washed platelets in *in vitro* study, CORM-3 (100 and 200 μM) inhibited thrombin-induced platelet aggregation. Similarly, antiplatelet effect was also observed when 3 mg/kg i.v. infusion of CORM-3 administered for 10 minutes in *ex vivo* study using rat. Interestingly, in presence of inhibitor of sGC (ODQ, 10 mg/kg, i.p.) and inhibitor of nitric oxide synthase (L-NAME, 30 mg/kg, i.p.), inhibition of thrombin-induced aggregation by CORM-3 was significantly blocked. Notably, in presence of inhibitor of K_{ATP} channel (glibenclamide, 10 mg/kg, i.p.) and p38 MAP Kinase (SCIO-469, 1 mg/kg, i.p.), inhibition of aggregation by CORM-3 was not blocked. In *in vivo* studies using animal models of thrombosis, we found that CORM-3-mediated antithrombotic effect was dependent on activation of sGC, NO and suppression of PAI-1 in arterial thrombosis and Arterio-Venous (A-V) shunt models. Therefore, we concluded that antithrombotic activity of CORM-3 may be mediated by activation of sGC, NO and inhibition of PAI-1.

1. Introduction

The use of CO as a potential therapeutic agent has emerged slowly due to its negative connotation as a toxic gas for mammalians since decades [1]. In the last few years' research on CO for the regulation of many physiological processes has been reported. Endogenously, CO is produced as a by-product during breakdown of heme moiety and this reaction is facilitated by the enzyme heme oxygenase (HO). HO exists mainly in inducible, HO-1 and constitutive, HO-2 forms. Heme breakdown by HO-1 produces bilirubin, iron (Fe++) and CO, out of which CO has been recognized as cytoprotective gas and is mimicking the role of HO-1 in many pathophysiological conditions [2]. Although the mechanism(s) underlying the cytoprotective actions of CO has not been elucidated, evidence suggests that this gas exerts some of its effects via activation of the guanylate cyclase/ cGMP pathway [3,4]. A water-soluble form of CO-releaser, tricarbonyldichloro (glycinato) ruthenium (II) (CORM-3), has been developed and demonstrated as cardioprotective agent [5,6]. Recently we have reported that cardioprotection by CORM-2 (a lipid soluble fast CO-releaser), is highly concentration-dependent, independent of coronary endothelium and cardioprotective effect might be attributed to the activation of K_{ATP} channel present on vascular smooth muscle cell (VSMC) [7].

Earlier study using CO as gas showed that exposure of minipigs to low concentration (160 and 185 ppm) CO significantly increased platelet aggregation. Elevating CO concentration to 420 ppm showed adhesions of shape-changed platelets on the arterial endothelium that was revealed under scanning electron microscopy [8]. However, later studies showed inhibition of platelet aggregation by CO. It has been reported that CO inhibited release of ADP and serotonin from platelet [9]. Both endogenously derived and exogenously applied CO inhibits platelet aggregation by stimulating the activation of sGC [10,11]. Emerging studies indicated that CO may also exert important protection against thrombosis. Further, CO mitigates platelet adhesion to endothelium in response to inflammation [12]. Furthermore, CO inhibits platelet aggregation and thrombosis following organ transplantation, and may contribute to the inhibition of platelet-dependent thrombosis following the induction of HO-1 in a rodent artery injury model [13,14]. Moreover, inhalation of CO rescues mice from lethal ischemic injury by preventing microvascular thrombosis and the accumulation of fibrin [15]. Recently, it has been observed that the

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absence of HO-1 in aortic allograft recipient mice resulted in 100% mortality within 4 days due to arterial thrombosis. In contrast, recipient mice normally expressing HO-1 showed 100% graft patency and survival [16].

Transition metal carbonyls have been shown to be a safe and effective means of transporting and releasing CO groups. Recent study showed that CORM-3, a water-soluble CO-releaser inhibited human platelets by a mechanism independent of sGC [17]. The water-soluble properties of CORM-3 (a fast CO releaser) suggest that this compound may have clinical utility. Accordingly, the goal of the present study was to determine whether the antiplatelet effects of CORM-3 demonstrated *in vitro* are also present *ex vivo* and *in vivo*. To this end, we utilized a well-established rat model of arterial thrombosis (platelet rich thrombus), artrio-venous thrombosis (mixed thrombus) and venous thrombosis (RBC rich thrombus) in which fundamental physiological variables that modulate thrombosis were carefully monitored and controlled. We tested the hypothesis that CORM-3 may prevent agonist-induced platelet aggregation *in vitro*. Furthermore we tested the role of sGC, NO, K_{ATP} channel and MAPK in CORM-3-mediated antiplatelet activity. To study antiplatelet potential of CORM-3 and its mechanism(s), we employed *in vitro* and *ex vivo* experiments using washed rat platelets. Further, we extended our observations into *in vivo* animal models of thrombosis and mechanism(s).

2. Materials and methods

2.1. Animals

Male wistar rats (250–300 g body weight) were used in the study. The animals were kept in individually ventilated cages in a room with controlled temperature (23 ± 2 °C), lighting (12:12 h light–dark cycle) and relative humidity (55 ± 10%). Animals had free access to standard rat chow and water. The protocol for use of animals for conducting these experiments has been reviewed and approved by the Institutional Animal Ethics Committee (IAEC).

2.2. Chemicals

CORM-2 was obtained from Sigma chemical. CORM-3 was synthesized and iCORM-3 (inactive CORM-3) was prepared as described previously [6]. Thrombin was also purchased from Sigma Chemical, USA and prepared in phosphate buffer (pH 7.4). Urethane was purchased from Sigma Chemicals, USA. FeCl3 was purchased from Hi-Media, India. Clopidogrel bisulfate was the generous gift from Zydus Cadila, India. In all experiments, CORM-3 was dissolved in water for injection. ODQ, glibenclamide and SCIO-469 were formulated in DMSO (0.5%) + saline. L-NAME, Clopidogrel and CORM-3 were prepared in water for injection. CORM-3 was inactivated (termed as iCORM-3) by dissolving it in PBS and leaving it at room temperature for 24 h [6].

2.3. Preparation of rat washed platelets for aggregation studies

Rat blood samples were colleted from retro-orbital route under light ether anesthesia in to the tubes containing 3.8% trisodium citrate. All blood samples were centrifuged at 200Xg for 20 min and platelet rich plasma (PRP) was carefully collected. PRP was again centrifuged at 800Xg for 10 minutes and supernatant was removed. The platelet pellet was resuspended and washed three times in CGS (0.12 M sodium citrate, 0.1 M dextrose, 0.1 M Nacl, pH 6.5). The final resuspension was in modified Tyrode's buffer (138 mM Nacl, 2.9 mM Kcl, 12 mM NaHCO3, 0.4 mM MgCl2, 5.5 mM dextrose, 0.36 mM NaH2PO4, 1.8 mM CaCl2, pH 7.4). The platelet count, conducted on a Cell Dyn 3700 (Abbott Diagnostics), was adjusted with Tyrode's buffer to 3×10^8 / ml. Platelet activation inhibitors were not used during platelet isolation due to their potential effect on signaling pathways involved in shear-induced platelet activation. Washed Platelet (WP) suspension was allowed to rest for 30 min at 37 °C before conducting experiments.

2.4. Measurement of platelet aggregation using thrombin as aggregating agent

Platelet aggregation studies were performed on a SpectraMax 190 microplate reader in 96-well, flat-bottomed, micro titer plates [18] using the SOFTmax Pro data acquisition software (Molecular Devices Corp., California, USA). A 180-μl volume of WP was placed in each well, followed by addition of 20 μl of thrombin. For *in vitro* studies, WPs were incubated with various concentrations of CORM-3 (50, 100 and 200 μM) for 2 minutes at 37 °C before addition of thrombin. Readings were taken every 1-minute over a 5-minute period at 405-nm wavelength. During the run time, the plate was incubated at 37 °C and was shaken vigorously in a shaking mode at the maximal speed available. All platelet aggregation studies were performed in triplicate. Change in optical density (OD) was measured by taking OD of buffer as blank. Aggregations were performed using a modest concentration of thrombin (0.5 IU/ml for rat platelets). % Aggregation was calculated using formula:

$$\%\ Aggregation = [(Initial\ OD - Final\ OD) / Initial\ OD] * 100$$

2.5. Experimental protocol for ex vivo platelet aggregation study in rats ($n = 10$)

Wistar rats were divided randomly on body weight basis in to seven groups as follows:

Group 1: Vehicle treated (0.5 ml/kg/min, i.v. infusion for 10 minutes, 0.5% DMSO + Saline).
Group 2: i CORM-3 (3 mg/kg/min, i.v.) for 10 minutes.
Group 3: CORM-3 (3 mg/kg/min, i.v.) for 10 minutes.
Group 4: ODQ (10 mg/kg, i.p.) before 30 min.+ CORM-3 (3 mg/kg/min, i.v.) for 10 minutes.
Group 5: L-NAME (30 mg/kg, i.p.) before 30 min.+ CORM-3 (3 mg/kg/min, i.v.) for 10 minutes.
Group 6: Glibenclamide (10 mg/kg, i.p.) before 30 min. + CORM-3 (3 mg/kg/min, i.v.) for 10 minutes.
Group 7: SCIO-469 (1 mg/kg, i.p.) before 30 min. + CORM-3 (3 mg/kg/min, i.v.) for 10 minutes.

Blood samples were obtained through the retro-orbital route under anesthesia in tubes containing 3.8% trisodium citrate. Washed platelets were prepared as described above and samples were subjected to thrombin-induced platelet aggregation (0.5 IU/ml) assay. % Aggregation was calculated.

2.6. FeCl3-induced arterial thrombosis model in rats

Animals (n = 10) were treated as per given protocol and then subjected to FeCl3-induced arterial thrombosis. FeCl3 -induced chemical injury was used as a model of arterial thrombosis as previously described [19]. Briefly, rats were anaesthetized with urethane (1.25 g/ kg, i.p.). A midline cervical incision was made on the ventral side of the neck, and left carotid artery was isolated. A 2 × 3 mm strip of Whatman filter paper No.#1 saturated with 35% (w/v) FeCl3 was kept on the carotid artery for 5 min. A temperature probe (Thermalert-TH8, Physitemp Instruments Inc., Clifton, N.J., USA) was placed distal to Whatman filter paper to monitor the temperature of carotid artery. A sudden fall in temperature (about 2 °C) was taken as an indication of cessation of blood flow as a consequence to thrombus formation. Time to occlusion (TTO) was defined as the time from FeCl3