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Regular Article

The procoagulant properties of purified fibrinogen concentrate are enhanced by carbon monoxide releasing molecule-2 ☆

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ABSTRACT

Introduction: Fibrinogen concentrate has been demonstrated to enhance coagulation in vitro and in several clinical settings of coagulopathy. We have recently demonstrated that carbon monoxide releasing molecule-2 (tricarbonyldichlororuthenium (II) dimer; CORM-2) enhances fibrinogen as a substrate for thrombin via an attached heme. The objective of this study was to determine if CORM-2 modified fibrinogen concentrate would enhance coagulation more effectively than CORM-2 naïve fibrinogen concentrate.
Materials and Methods: In the first series of experiments, fibrinogen concentrate (final concentration 300 mg/dl) was exposed to 0, 50 or 100 μM CORM-2 for 5 min at 37 °C prior to being added to citrated, fibrinogen depleted plasma. In another series of experiments, citrated plasma obtained from 12 normal subjects was 50% diluted with crystalloid to which was added fibrinogen concentrate (final concentration 300 mg/dl) exposed to 0 or 100 μM CORM-2. Coagulation was activated with tissue factor (n = 8 per condition). Thrombus growth was monitored with thrombelastography for 15 min.
Results and Conclusions: CORM-2 modification of fibrinogen concentrate significantly enhanced the velocity of clot formation (30-50%) and strength (15-31%) in fibrinogen deficient plasma. Similarly, while diluted plasma-derived thrombi demonstrated a marked decrease in velocity of formation (54%) and strength (61%), fibrinogen concentrate significantly enhanced velocity (217%) and strength (171%); however, CORM-2 modified fibrinogen concentrate significantly increased velocity (303%) and strength (205%) to a greater extent. Additional *in vitro* investigation and *in vivo* preclinical assessments of the hemostatic efficacy of CORM-2 modified fibrinogen concentrate are warranted.

Introduction

The continuing need for quickly accessible, durable blood components to treat coagulopathy in surgical/military settings is the focus of several investigators worldwide. One such therapeutic that is well established in Europe and recently approved in the United States is human fibrinogen concentrate (RiaSTAP®, CSL Behring GmbH, Marburg, Germany), a product derived from cryoprecipitate that is stable at room temperature (range 2-25 °C) for up to 30 months. Fibrinogen concentrate has been demonstrated to improve clot strength in human blood diluted with either crystalloid or colloidal volume expanders [1,2]. In a preclinical, porcine model of dilutional coagulopathy, fibrinogen concentrate improved the velocity of thrombus formation and strength [3]. Retrospective studies of severe hemorrhage [4,5] and cardiopulmonary bypass [5,6] demonstrated reduced need for red blood cell (RBC), fresh frozen plasma (FFP) and platelet transfusion and reduced bleeding following administration of fibrinogen concentrate; however, no untreated patients with hemorrhage were used for comparison. Finally, in a prospective investigation with patients undergoing coronary artery bypass surgery [7], prophylactic administration of fibrinogen concentrate decreased bleeding without significant postoperative hypercoagulability (e.g., vein graft occlusion). In sum, fibrinogen concentrate provides a quickly employable, effective treatment of congenital or acquired hypofibrinogenemia-associated coagulopathy.

While fibrinogen concentrate administration is an effective therapeutic intervention, recent observation concerning fibrinogen biochemistry raised the possibility that this product could be further enhanced. We recently reported that of carbon monoxide releasing molecule-2 (tricarbonyldichlororuthenium (II) dimer; CORM-2) enhances plasmatic coagulation [8], primarily by enhancing fibrinogen as a substrate [9]. Electron microscopy revealed that CORM-2 exposure changed the micromorphology of thrombi, resulting in a decrease in thick fibrin polymer formation in favor of thin fiber production [10]. Finally, we recently determined that the molecular mechanism by which carbon monoxide modifies fibrinogen is via an

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attached heme group that putatively changes the three dimensional configuration of the molecule [11]. Taken as a whole, it seemed possible that carbon monoxide exposed fibrinogen concentrate might be more biologically active than unexposed fibrinogen.

The purpose of the present investigation was to determine if the substrate efficiency of fibrinogen concentrate could be enhanced by CORM-2 exposure. This hypothesis was subsequently tested with thrombelastography utilizing fibrinogen deficient and hemodiluted plasmas.

Materials and methods

Fibrinogen deficient plasma experiments

Fibrinogen deficient plasma anticoagulated with sodium citrate was obtained from a commercially available source (Affinity Biologicals, Inc., Ancaster, Ontario, Canada). The plasma had a prothrombin time >120 sec, an activated partial thromboplastin time of >300 sec, a 147% normal prothrombin activity, a 104% normal factor V activity, a 142% normal factor VIII activity, and a fibrinogen concentration of 40 mg/dl as determined by enzyme linked immunosorbant assay. Fibrinogen concentrate was reconstituted with dH_2O to a approximate concentration of 20 mg/ml. Based on the range of constituents by weight of the lyophized product (e.g., fibrinogen 900–1300 mg, albumin 400–700 mg, L-arginine 375–660 mg, NaCl 200–350 mg, sodium citrate 50–100), we estimated that on average 44.25% of the concentrate was fibrinogen and diluted the product accordingly. Fibrinogen concentrate was provided as a generous gift from CSL Behring. The reconstituted fibrinogen concentrate was exposed to 0, 50, or 100 μM CORM-2 in dimethyl sulfoxide (Sigma-Aldrich, Saint Louis, MO, USA), with the addition of CORM-2 or vehicle accounting for 1% of the final mixture at least 5 min prior to mixing with fibrinogen deficient plasma. The samples exposed to 50 and 100 μM CORM-2 were noted to have some precipitate that quickly dissolved over 2–3 min.

The final volume for plasma sample mixtures was 360 μl. Sample composition consisted of 287 μl of plasma and 43 μl of fibrinogen concentrate – this combination should have yielded a final fibrinogen concentration of 300 mg/dl (40 mg/dl from the plasma, 260 mg/dl from the fibrinogen concentrate). The final concentration of CORM-2 was 12 μM, a concentration that would not affect coagulation of unexposed plasma-derived fibrinogen based on previous results [12]. Other mixture additions included 10 μl of tissue factor reagent (0.1% final concentration in dH_2O; Instrumentation Laboratory, Lexington, MA, USA), and 20 μl of 200 mM $CaCl_2$.

Hemodilution experiments

Individual normal subject plasma (George King Bio-Medical, Overland Park, KS, USA; 5 male, 7 female, aged 19–50 yo) anticoagulated with sodium citrate was utilized for experimentation. The plasma was either: 1) undiluted; 2) diluted 50% with Isolyte®, a calcium-free electrolyte solution with pH 7.4 (B. Braun Medical Inc., Irvine, CA, USA); 3) diluted 50% with Isolyte® and a volume of fibrinogen concentrate to increase the fibrinogen concentration by 300 mg/dl; and finally, 4) samples were diluted 50% with Isolyte® and a volume of fibrinogen concentrate exposed to 100 μM CORM-2 to increase the fibrinogen concentration by 300 mg/dl.

Another sealed fibrinogen concentrate was reconstituted with 50 ml of sterile H_2O as per the manufacturer's instructions, yielding a final concentration of 23 mg/ml. This solution was exposed to 0 or 100 μM CORM-2. CORM-2 addition as a single bolus resulted in significant precipitation that did not clear with time; however, when added as 5 20% additions, nearly imperceptible precipitation was observed. Our previous work has demonstrated that exposure of purified fibrinogen (e.g., without albumin, etc.) to CORM-2 resulted in insoluble precipitates, presumably from spontaneous polymerization [9].

As with the previous series of experiments, the plasma sample mixture volume was 360 μl. Undiluted samples consisted of 330 μl of plasma; 50% diluted samples were composed of 165 μl of plasma and 165 μl Isolyte®; 50% diluted samples with fibrinogen concentrate addition were composed of 165 μl of plasma, and 122 μl Isolyte® and 43 μl of fibrinogen concentrate; lastly, 50% diluted samples with CORM-2 exposed fibrinogen concentrate addition were composed of 165 μl of plasma, and 122 μl Isolyte® and 43 μl of fibrinogen concentrate. As with the previous experimental series, concentration of CORM-2 was 12 μM, a concentration that would not affect coagulation of unexposed plasma-derived fibrinogen based on previous results [12]. All mixtures subsequently had 10 μl of tissue factor reagent (0.1% final concentration in dH_2O), and 20 μl of 200 mM $CaCl_2$.

Thrombelastographic analyses

Plasma sample mixtures were placed in a disposable cup in a computer-controlled thrombelastograph® hemostasis system (Model 5000, Haemoscope Corp., Niles, IL, USA), with addition of $CaCl_2$ as the last step to initiate clotting. Data were collected for 15 min. Thrombelastographic variables measuring clot initiation, velocity and strength were recorded at 37 °C as previously described [8,9,11] and are displayed in Fig. 1. The nomenclature used to describe these phenomena is as follows: ***Time to maximum rate of thrombus generation (TMRTG):*** This is the time interval (sec) observed prior to maximum speed of clot growth. ***Maximum rate of thrombus generation (MRTG):*** This is the maximum velocity of clot growth observed (dynes/cm^2/sec). ***Total Thrombus Generation (TTG):*** This is the total area under the velocity curve during clot growth (dynes/cm^2), representing the amount of clot strength generated during clot growth.

Statistical analyses

Thrombelastographic data are presented as mean + SD in the first series of experiments and as individual parameter value changes over time with associated condition parameter value means. All conditions were represented with n = 8 or greater replicates/individuals as this provides a power ≥0.8 with $P<0.05$ [8,9,11]. Analyses of the effects of CORM-2 mediated enhancement of fibrinogen concentrate-dependent coagulation in fibrinogen deficient plasma were conducted with one way analysis of variance (ANOVA) with the Holm-Sidak *post hoc* test (SigmaStat 3.1, Systat Software, Inc., San Jose, CA, USA).

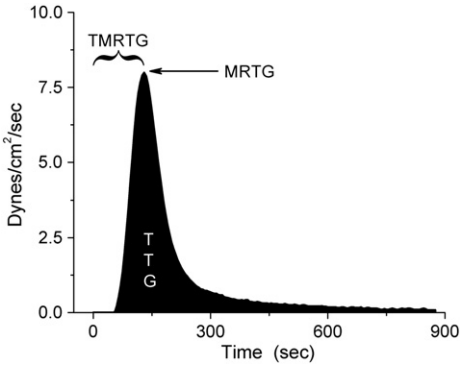

Fig. 1. Thrombelastographic Variables. A normal thrombus growth velocity curve with labeled parameters is displayed. TMRTG = Time to maximum rate of thrombus generation: This is the time interval (sec) observed prior to maximum speed of clot growth. MRTG = Maximum rate of thrombus generation: This is the maximum velocity of clot growth observed (dynes/cm^2/sec). TTG = Total Thrombus Generation: This is the total area under the velocity curve during clot growth (dynes/cm^2), representing the amount of clot strength generated during clot growth.