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Regular Article

Peroxynitrite – altered platelet mitochondria—A new link between inflammation and hemostasis

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SUMMARY

Using porcine blood, we tested the hypothesis that peroxynitrite ($ONOO^-$) may affect platelet-fibrin clot formation, clot retraction rate (CRR) and fibrinolysis through the inhibition of platelet energy production. It was found that $ONOO^-$ reduces CRR and enlarges final clot size in platelet rich plasma (PRP) (IC_{50} = 100 μM) and in whole blood (IC_{50} = 200 μM) dose-dependently. In a reconstituted system (washed platelets + fibrinogen), CRR was inhibited by 5–100 nM $ONOO^-$ (IC_{50} = 25 nM). Concentrations of $ONOO^-$ reducing CRR in PRP, inhibited platelet oxygen consumption, augmented lactate production and decreased total ATP contents in clots derived from PRP. In washed platelets $ONOO^-$ (5–20 nM) produced a drop of the mitochondrial transmembrane potential ($\Delta\Psi_m$). Blocking of mitochondrial energy production resulted in a reduction of CRR, whereas inhibition of glycolysis failed to affect CRR. $ONOO^-$, up to 300 μM, failed to affect coagulation in platelet free plasma. Fibrinolysis of platelet-fibrin clots was enhanced by $ONOO^-$ (25–300 μM), cytochalasin B and following the reduction of platelet energy production. Fibrinolysis of plasma clots was resistant to $ONOO^-$ treatment up to a concentration of 500 μM. Tromboelastometry (ROTEM) measurements performed in PRP show that inhibition of platelet energy production or treatment with $ONOO^-$ (100–300 μM) diminishes MCF, alpha angle and MCE parameters. Blockage the platelet contractile apparatus by cytochalasin B resulted in reduction of CRR and ROTEM variables (MCF, alpha angle, MCE). We conclude that physiologically relevant $ONOO^-$ concentrations may inhibit clot retraction, reduce clot stability and accelerate its lysis through the inhibition of platelet mitochondrial energy production.

Introduction

An accumulation of experimental, clinical, and epidemiological studies has revealed the importance of the cross-talk between inflammation and coagulation in the pathogenesis of hemostatic abnormalities in humans [1–3]. Currently, it is accepted that inflammation positively correlates with thrombosis, yet there are inflammation associated diseases, such as sepsis, where hemorrhage can also emerge [4,5]. The mechanism(s) linking hemostasis abnormalities with inflammation are not completely understood.

Studies on the link between inflammation and thrombosis have thus far been focused on long-term, cytokines-mediated mechanisms [6–8]. Much less is known about the role of substances used to kill pathogens which can emerge in large quantities near the activated inflammatory cells almost immediately after initiation of inflammation. The group of such compounds comprises mainly reactive oxygen and nitrogen species and hypochlorous acid [7,9,10]. It is well documented that prolonged bacterial or viral infection may result in a dramatic rise in the quantity of such substances released into blood. A prominent example is peroxynitrite ($ONOO^-$), formed in a very rapid reaction from NO and $O_2^{\cdot-}$, produced by activated inflammatory cells. In humans, peroxynitrite generation represents a crucial pathogenic mechanism in hemostasis abnormalities associated with myocardial infarction, type 1 diabetes, stroke, sepsis, rheumatoid arthritis, ischemia and reperfusion of cardiac muscle, and hypercholesterolaemia [7].

So far studies performed on the effect of $ONOO^-$ on hemostasis have been focused on its direct effects on clotting factors and platelets [11–15]. However, accumulated evidence indicates that concentrations of $ONOO^-$ much lower than those which alter clotting factors activity can suppress mitochondrial energy production. It has been demonstrated that muscle cells from patients with sepsis, where production of NO and $O_2^{\cdot-}$ is known to be strongly elevated [16–18], have impaired mitochondria and produce less ATP in comparison to normal control. This observation may indicate that $ONOO^-$ formed in close proximity to activated inflammatory cells may affect hemostasis through the inhibition of platelet energy production. Such a hypothesis seems likely since one of the important steps in hemostasis - clot retraction - critically depends on platelet energy production [19]. Clot retraction has been reported to have a great impact on the mechanical properties of thrombus (stability and elasticity) and on its lysis [20–22].

The properly retracted clot has smaller volume which permits faster recanalization of an occluded (by thrombus) blood vessel,

Abbreviations: CRR, clot retraction rate; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; CCCP, Carbonyl cyanide 3-chlorophenylhydrazone; TMRM, tetramethylrhodamine methyl ester.

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which results in shortening the time of ischemia. The retracted clot is more strongly connected with a vessel wall (which decreases the risk of thromboembolic events) and is less susceptible to fibrinolysis (which reduces the risk of bleeding) [20,23,24].

Abnormalities in clot retraction may thus contribute to both the pathogenesis of thromboembolic events and a tendency toward bleeding.

Consequently, these studies were undertaken to establish whether peroxynitrite may affect platelet-fibrin clot formation, its clot retraction, and fibrinolysis through the inhibition of platelet energy production.

Materials and methods

Chemicals

Recombinant tissue plasminogen activator (Actylise) was purchased by Boehringer Ingelheim GmbH (Ingelheim, Germany). Chrono-lume (luciferin-luciferase mix) was purchased by Chrono-log (Havertown, PA, U.S.A.). Tetramethylrhodamine methyl ester (TMRM) was purchased by Invitrogen (Carlsbad, CA, U.S.A.). Other chemicals were from Sigma Chemical Co (St. Louis, MO, U.S.A).

Synthesis of $ONOO^-$

Peroxynitrite was synthesized and purified as described by Koppenol et al. [25]. Stock solutions containing at least 200 mM $ONOO^-$ were prepared and stored at −70 °C. The concentration was determined prior to each experiment by measuring the absorbance at 302 nm ($\varepsilon_{302} = 1670\ M^{-1}\ cm^{-1}$). Decomposed $ONOO^-$ was obtained by allowing the compound to decay in an 0.5 M phosphate buffer (pH 7.4) at 25 °C for 30 min.

Animals

A total of 50 domestic pigs (breed: Polish Large White) of both sexes, approximately 9 months old, with a mean weight of 90–100 kg, were used in the experiments. The animals were raised on local farms under normal agricultural husbandry conditions. Pigs were fed the grower chow diet consisting of grain (rye, barley, wheat, maize) and soybean. The animals for this study were selected at random from available litters. Prior to blood collection, the pigs were starved for at least 12 hours. The study protocol and procedures were approved by the Ethics Committee at the Medical University of Białystok.

Blood collection

Blood collection was performed in a local slaughterhouse (PMB, Bialystok, Poland). Forty milliliters of blood was withdrawn by direct carotid catheterization and collected into 3.8% (w/v) sodium citrate, one volume per nine volumes of blood. To avoid contamination of blood by tissue factor the initial 10 ml of blood was discarded. Platelet number in the blood of studied animals varied from 4.6×10^8/ml to 6.5×10^8/ml. Platelet number in PRP was adjusted to 4×10^8/ml with autologous plasma before each experiment.

Platelet preparation

Platelet rich plasma (PRP) was obtained by centrifugation of whole blood at 200 ×g for 20 min. To prepare washed platelets, PRP was acidified to pH 6.5 with 1 M citric acid, the suspension was centrifuged at 1500 ×g for 20 min to obtain a pellet which was resuspended in a Ca^{2+}-free Tyrode-Hepes buffer (152 mM NaCl, 2.8 mM KCl, 8.9 mM $NaHCO_3$, 0.8 mM KH_2PO_4, 0.8 mM $MgCl_2$, 5.6 mM glucose, apyrase (2 U/ml), 10 μM EGTA, BSA (3.5 mg/ml) and 10 mM Hepes, pH 6.5, osmolarity of 340 mOsm). The platelets were washed once with the above buffer and finally suspended in the same buffer with the exception that, in the final suspension medium, apyrase and EGTA were omitted and pH was adjusted to 7.4. The platelet concentration was standardized to 4×10^8 cells/ml by dilution with Tyrode-Hepes buffer. Platelet number was determined using a Coulter® Hematology Analyzer (Beckman Coulter Inc., U.S.A.).

Measurement of kinetics of clot retraction

The kinetics of clot retraction was evaluated by the method described by Osdoit and Rosa [26]. In brief, measurements were performed in glass tubes (12×75 mm) containing a cushion of polymerized polyacrylamide, 6% (w/v), at the bottom to avoid clot adherence. Prior to measurement, the tubes were rinsed extensively with Tyrode–Hepes buffer. Aliquots (0.4 ml) of whole blood or platelet rich plasma (PRP) were added to 3.1 ml T-H buffer (pH 7.4), containing 2.5 mM $CaCl_2$, preheated to 37 °C, and clot retraction was initiated by gently mixing the suspension. In experiments with washed platelets 0.4 ml of cell suspension (4×10^8 cells/ml) were added to 3.1 ml of T-H buffer containing bovine fibrinogen (2 mg/ml) and 2.5 mM $CaCl_2$ The retraction was initiated by the addition of thrombin (final conc. 1 U/ml). The fibrinogen was previously dialyzed in a platelet resuspension buffer to eliminate sodium citrate. The reaction was developed at 37 °C. Pictures were taken for one hour at 10 min intervals and after 120 min using a digital camera. Quantification of retraction was performed by assessment of the clot area by use of Motic Images Plus 2.0 ML software, and data were processed using Excel 11. Clot surface areas were plotted as a percentage of maximal retraction (*i.e.* volume of platelet suspension). Data was expressed as follows: percentage of retraction = ((area t_0-area t)/area t_0)×100.

Thromboelastometric (ROTEM) analyses

ROTEM technology is described elsewhere [27]. Thromboelastometric measurements were performed using ROTEM system (Tem International GmbH, Manheim, Germany). We measured the following parameters: CT (clotting time) – time from start of measurement to the beginning of the fibrin polymerization process; alpha angle (α) – the angle showing the dynamics of clot formation, MCF (maximum clot firmness) – a parameter reflecting the strength of the formed clot to resist the pin oscillation; and MCE (maximum clot elasticity) – a parameter reflecting the flexibility of the clot. MCF and MCE can be treated as parameters describing the stability of a clot and its susceptibility to reversible (elastic) deformation. All ROTEM measurements were performed by the same experienced operator as follows: 0.3 ml of citrated PRP was transferred into a preheated cup containing 20 μl of 0.2 M calcium chloride as activator and repeatedly gently pipetted to mix the components.

Determination of fibrin polymerization profiles and fibrin plasma clot lysis by the turbidimetric method

The kinetics of fibrin formation and plasma clot lysis was evaluated by the turbidimetric method described in details by Carter et al. [28]. The following variables were determined from the turbidimetric clotting assay curve: lag time (Lag_C), which represents the time at which sufficient protofibrils have formed to enable lateral aggregation; maximum absorbance (MaxAbsC) which reflects the degree of fibrin cross linking and clotting rate (CR) The intra-assay coefficients of variation for the calculated parameters were 5.2 and 7.7% for thrombin and the calcium chloride activation mixture respectively.

To evaluate of fibrin plasma clot lysis, assay buffer was supplemented with rtPA (2.08 μg/ml). The following variables were determined from the clot lysis curvetime to complete lysis, (Lys_t)partial lysis time ($Lys50_{MA}$) and a lysis rate (LR) The intra-assay coefficient of variation for the calculated parameters was 7.8%.

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