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Regular Article

Peroxynitrite may affect clot retraction in human blood through the inhibition of platelet mitochondrial energy production

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ABSTRACT

Peroxynitrite ($ONOO^-$) contributes to hemostasis abnormalities associated with inflammatory states by a poorly understood mechanism. Here we show that $ONOO^-$ may affect clot retraction (CR), an important step in hemostasis, by reducing contractility of human platelets resulting from the inhibition of mitochondrial energy production. Reduced CR may result in thromboembolic and hemorrhage events. The results show that in human blood, *in vitro*, physiologically relevant $ONOO^-$ concentrations reduce clot retraction rate and enlarge final clot size. The stressor was more effective in reconstituted system consisting of washed platelets and fibrinogen, (IC_{50} = 25 nM) than in platelet rich plasma (IC_{50} = 75 μM) or in whole blood (IC_{50} = 120 μM), indicating that its effect depends on the number of targets. Retardation of CR by lower concentrations of $ONOO^-$ resulted in reduction of platelet energy production due to impairment of mitochondria but not from tyrosine nitration or inhibition of actin polymerization. In washed platelets nanomolar $ONOO^-$ concentrations produced a drop of the mitochondrial transmembrane potential ($\Delta\Psi_m$) explaining high sensitivity of CR (a large consumer of platelet energy) to stressor. Thromboelastometry measurements showed that $ONOO^-$ may diminish clot stability and elasticity through the reduction of platelet contractility. Our findings suggest that in humans $ONOO^-$- altered platelet mitochondria represent a new link between inflammation and hemostasis.

Introduction

In response to inflammation, mammalian immune cells form high quantities of NO and superoxide which are used to kill pathogens [1]. Neither superoxide nor NO is toxic *in vivo* to host cells because there are efficient means to minimize their accumulation [1–3]. However, when both superoxide and NO are synthesized within a few cell diameters of each other – a condition likely to take place in the blood of subjects with an inflammatory state – they will rapidly combine to form peroxynitrite ($ONOO^-$) [1,4,5]. Due to the extremely low stability of $ONOO^-$ in aqueous buffers (half-life ~1 s) its distribution in blood is nonhomogenous. Thus, under inflammatory conditions, the highest amounts of $ONOO^-$ are expected to be present in close proximity to stimulated macrophages and dysfunctional endothelial cells, where the local concentrations of a stressor were estimated to reach about 1 mM [6] and 0.5 μM respectively [7].

The cytotoxic action of $ONOO^-$ is related to its ability to peroxidate lipids, alter DNA structure, and oxidize protein sulfhydryls and nitrate tyrosine residues in a variety of proteins [1,2,5,8].

Accumulating evidence support the view that local formation of peroxynitrite from superoxide and NO, generated by activated inflammatory cells in the vicinity of the vascular endothelium, represents a crucial pathogenic mechanism in serious hemostasis abnormalities associated with myocardial infarction, diabetes, stroke, sepsis, hypercholesterolaemia and atherosclerosis [1,5,9]. The detailed mechanism(s) by which peroxynitrite affects hemostasis remain unclear.

So far performed studies indicate that relatively high (micromolar–milimolar) concentration of this stressor can affect clotting factors (mainly through the nitration of tyrosine) and platelets [10–14]. However, numerous experimental studies on the effect of peroxynitrite on cellular energetics have established that much lower (nanomolar) concentrations of $ONOO^-$ can suppress mitochondrial energy production. Notably, $ONOO^-$ readily inactivates mitochondrial enzymes involved in oxidative metabolism, alter mitochondrial calcium homeostasis, reduce mitochondrial transmembrane potential ($\Delta\Psi_m$), and promote the opening of the permeability transition pore [1,15,16].

In porcine platelets, peroxynitrite has been reported to inactivate mitochondrial enzymes involved in oxidative metabolism [14]. In patients with septic shock (a clinical condition characterized by impaired hemostasis) a strong positive correlation between decreasing platelet mitochondrial functionality and disease severity was reported [17]. Similarly, more recent studies also demonstrate that in platelets from patients with SIRS (Systemic Inflammatory Response Syndrome), clinical condition associated with abnormal peroxynitrite production and impaired hemostasis [1], decrease of $\Delta\Psi_m$ significantly correlates

Abbreviations: CRR, clot retraction rate; ROTEM, rotational thromboelastometry; CT, clotting time; MCF, maximum clot firmness; PRP, platelet-rich plasma; ODQ, H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one; CCCP, Carbonyl cyanide 3-chlorophenylhydrazone; TMRM, tetramethylrhodamine methyl ester.

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with the severity of a disease [18]. It is therefore possible that at pathological conditions $ONOO^-$, 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 [19,20].

Clot retraction (CR) is defined as the slow shrinking of a freshly formed platelet aggregate (attached to an injured blood vessel wall) reinforced by the fibrin strands connecting neighboring cells [21–23]. Platelet-fibrin clot suppresses bleeding and serves as a temporary extracellular matrix in the wound area [23]. The physiological role of CR is to reduce the clot volume which facilitates recanalization of an occluded, by thrombus, blood vessel. Faster vessel recanalization results in shortening the time of ischemia of neighboring tissue. A properly retracted clot is strongly connected with the vessel wall and mechanically more stable [21]. An unstable clot, after detaching, may cause thromboembolic events. *In vivo*, clot retraction may thus regulate the size and stability of forming thrombi, and its abnormalities may contribute to both the pathogenesis of thromboembolic events and a tendency toward bleeding.

Consequently, we initiated studies to establish whether peroxynitrite can affect hemostasis through the inhibition of CR resulted from impaired platelet mitochondria. Our preliminary studies performed on a porcine blood model confirmed such a possibility [20]. We now report, for the first time, that in human blood physiologically relevant peroxynitrite concentrations may inhibit retraction and stability of the clot due to inhibition of energy production in platelet mitochondria *via* a mechanism not necessarily related to tyrosine nitration.

Materials and methods

Chemicals

Polyclonal rabbit anti-3-nitrotyrosine antibody and monoclonal HRP-conjugated goat anti-rabbit IgG were purchased Santa Cruz Biotech (Santa Cruz, CA, U.S.A.) Enhanced luminescence Super Signal West Pico Substrate was from Thermo Fisher Scientific (Waltham, MA, U.S.A.). 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.). Collagen (fibrillar, from equine tendon) was from Hormon Chemie (Munich, Germany). Tirofiban (Aggrastat) was from Merck Sharp & Dohme Idea Inc. (Glattbrugg, Switzerland). Other chemicals were from Sigma Chemical Co (St. Louis, MO, U.S.A).

Blood collection and platelet preparation

Venous blood was collected from healthy volunteers with minimum trauma and stasis via a 21-gauge needle (0.8 × 40 mm) into 10 ml polypropylene tubes containing 1 ml of 130 mM trisodium citrate. All procedures were conducted in accordance with the principles of Declaration of Helsinki and the study was approved by the local Ethics Committee on human research. 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, supplemented with apyrase (2 U/ml) and 1 μM PGE_1, and 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, 0.2% BSA and 10 mM Hepes, pH 7.4, osmolarity of 300 mOsm). To some experiments, the platelets suspension was next passed through a chromatographic column filled with Sepharose 2B using Tyrode-Hepes buffer as an eluent. Collected platelets were standardized to 2×10^8 cells/ml by dilution with Tyrode-Hepes buffer. Platelet number was determined using Coulter® Hematology Analyzer (Beckman Coulter, Fullerton, CA).

Synthesis of $ONOO^-$

Peroxynitrite was synthesized by the reaction of acidified H_2O_2 (1.4 M) with $NaNO_2$ (1.2 M) in a quenched flow reactor [24]. The excess of H_2O_2 was removed by passage the product over column filled with a granular manganese oxide (IV). Stock solutions containing at least 200 mM $ONOO^-$ were collected and stored at -70 °C. The concentration was determined prior to each experiment by measuring the absorbance at 302 nm ($\varepsilon_{302} = 1670\ M^{-1}\ cm^{-1}$). Typically used $ONOO^-$ concentrations did not caused an increase of pH of the samples. Control experiments were carried out with decomposed $ONOO^-$, obtained by allowing the compound to decay in 0.5 M phosphate buffer (pH 7.4) at 25 °C for 30 min.

Measurement of kinetics of clot retraction

Measurement of the kinetics of clot retraction in PRP and whole blood were performed in non-siliconized glass tubes (12 × 75 mm) containing a cushion of polymerized polyacrylamide, 6% (w/v), at the bottom to avoid clot adherence. Prior to measurements tubes were rinsed extensively with Tyrode–Hepes buffer. Aliquots (0.4 ml) of whole blood or PRP were added to 3.1 ml of T-H buffer (pH 7.4), containing 2.5 mM $CaCl_2$, preheated to 37 °C, and clot retraction was initiated by gently mixing of the suspension The kinetics of clot retraction in artificial system consisting of washed platelets, bovine fibrinogen (2 mg/ml final conc.) and thrombin from human plasma (1 U/ml final conc.) was evaluated by the method described in details by Osdoit and Rosa [25]. 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 clot area by use of the Motic Images Plus 2.0 ML (Motic, China) software, and data were processed using Microsoft Excel 11. Clot surface areas were plotted as percentage of maximal retraction (*i.e.* volume of platelet suspension). Data were expressed as follows: percentage of retraction (relative clot volume) = ((area t_0 − area t)/area t_0) × 100. In experiments, when exogenous ATP was used, washed platelets were permeabilized with saponin, typically 5 μg per each 10^8 platelets. This concentration was reported to enable aggregation of platelets in the presence of exogenous inositol triphosphate (which has similar molar weight to ATP) [26]. This concentration of saponin also provided aggregation of washed platelets in the presence of extracellular calcium (1 mM).

Measurement of ATP content in retracted clots

Clots derived from standardized (2×10^8 cells/ml) PRP samples formed during one hour incubation at 37 °C were carefully transferred with plastic Pasteur pipette to 3 volumes of ice cold 6% (w/v) perchloric acid, sonicated and left at 0 °C for 20 min. The extracts were centrifuged to remove proteins and neutralized with ice cold 6 M KOH/0.5 M morpholine sulphonic acid. ATP content was determined in neutralized cellular extracts by the luciferase-luciferin assay [27].

Measurement of the respiration rate

Oxygen consumption was measured polarographically with a Clark-type oxygen electrode, (model YSI 5300A, YSI Life Sciences, U.S.A.), in a closed vessel (YSI sample micro chamber) of 1 ml at 37 °C.

Measurement of lactate production in clotting PRP

Aliquots of clotting suspensions prepared as described above (in the section "Measurement of kinetics of clot retraction") were incubated at 37 °C in glass tubes. Incubation was started by the addition of glucose to the final concentration of 10 mM and was carried out for 60 min. It was stopped by the addition of 3 volumes of cold 6% (w/v) perchloric acid.