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Oxidative stress in sepsis. Possible production of free radicals through an erythrocyte-mediated positive feedback mechanism

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ABSTRACT

Background: Sepsis is an illness with a high morbidity for which no effective treatment exists. Its treatment has a high cost because it usually requires an intensive care unit and expensive antibiotics. The present study focus in the production of reactive oxygen species in the early stages of sepsis. This study aimed at investigating the production of reactive oxygen specie during the inflammatory response in patients with sepsis.
Methods: Reactive oxygen specie production and insoluble myeloperoxidase obtained from fresh whole blood were measured by photon counting chemiluminescence in the blood of 18 septic patients and 12 healthy individuals. Modified red blood cells were evaluated by staining of blood smears. The production of reactive oxygen species by macrophages and polymorphonuclear leukocytes put into contact with modified red blood cells were also assessed by photon counting chemiluminescence.
Results: The appearance of oxidatively modified erythrocytes, which is an evidence of oxidative stress, was supported by the detection of reactive oxygen species and insoluble myeloperoxidase in the whole blood of all septic patients. Peroxynitrite was the main reactive oxygen species found in the whole blood. Oxidatively modified erythrocytes activated phagocytic cells *in vitro*, leading to the considerable production of free radicals.
Conclusion: It was found that sepsis led to a high oxidative stress and to extensive modification of erythrocytes. It is proposed that a positive feedback mechanism, involving the activation of circulating leukocytes by these modified erythrocytes would maintain the pro-oxidative state even after the disappearance of bacteria.

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Introduction

Sepsis is widely recognized as a life-threatening organ dysfunction caused by dysregulated host response to infection.[1] Sepsis is the leading cause of death in non-cardiac intensive care units around the world. Death rates are high, with 20% for sepsis, 40% for severe sepsis, and more than 60% for septic shock.[2] Thus, it is essential to clarify the mechanisms that lead to lethality in this disease, so that they may become targets for therapies.

For most bacterial infections, polymorphonuclear neutrophils (PMNs) represent the first line of defense of the innate immune system. Phagocytosis of the microorganisms induces apoptosis in PMNs, which is dependent upon reactive oxygen species (ROS) production and is important for the resolution of infection and inflammation.[3–5]

Recently, studies of sepsis have expanded their focus to include microparticles formed as a result of PMN-derived insoluble myeloperoxidase (iMPO) endothelial and platelet cell activation. The resulting production of ROS, mainly hypochlorous acid generated by myeloperoxidase (MPO), impairs endothelial vascular function *via* increased oxidative stress and effects on the coagulation cascade.[6,7]

The present study sought to investigate the production of ROS during the inflammatory response in septic patients using chemiluminescence in real time.

Materials and methods

Blood donors and ethical considerations

This study was approved by the Ethics Committee of the Complexo Hospitalar Universitário Professor Edgard Santos in Salvador, Brazil. During the period from March 2010 to December 2010, blood samples were collected from 12 healthy volunteers at the hospital and from 18 patients who were diagnosed with sepsis/septic shock and received antibiotic therapy in the intensive care unit. Informed consent was either sought from the patients, or, when not possible, from their next of kin.

Measurement of ROS in whole blood samples

ROS levels in fresh whole blood samples were measured by chemiluminescence using a sensitive photon counter,[8] with L-012 (Wako Pure Chemical Industries, Osaka, Japan) used as a secondary emitter. Whole blood samples without any prior separation were added to Petri dishes and diluted with Hanks' balanced salt solution in a ratio of 1:1. After baseline reading for 200 s, 50 μM of L-012 was added to the plate and reading done after each sample reaches its plateau of photons emitted by L-012 activated by ROS for 100 s. The effects of ROS were characterized using specific inhibitors, including 250 μM of hydralazine and 1 mM of desferrioxamine as inhibitors of peroxynitrite ($ONOO^-$) and 9.4 μM of superoxide dismutase (SOD) as inhibitor of superoxide and 40 μM of sodium azide as inhibitor of MPO.

Detection of iMPO activity

For microparticle analysis, were collected 10 mL of peripheral blood with heparin, donated by septic patients and healthy volunteers. The separation of plasma and erythrocytes was performed by centrifugation of whole blood at $290 \times g$ for 10 min at 4 °C. Then, the platelet-rich plasma (PRP) was centrifuged at $4500 \times g$ for 5 min at 4 °C. The supernatant was subjected to three subsequent ultracentrifugation $100{,}000 \times g$ at the TLA-100.3 rotor (Beckman Instruments Inc., Palo Alto, CA, USA) for 45 min at 4 °C. After ultracentrifugation the supernatant was discarded and the pellet containing the microparticles with MPO or iMPO was resuspended in Hank's balanced salt solution (HBSS).

MPO has strongly cationic loads and isoelectric point >10,[9] and due to this characteristic, it is believed that MPO would be able to directly associate the microparticles by electrostatic interactions.[10,11]

MPO activity was assessed by estimating HOCl production using a previously described chemiluminescence method.[12] Samples (resuspended pellet containing MPO) with known amounts of protein were placed in dishes, sealed with cling film and maintained at 37 °C in a thermostatic light-sealed chamber. Their chemiluminescence emissions were then collected by reflections off a concave mirror and focused onto the photomultiplier tube. HOCl production derived for myeloperoxidase-hydrogen peroxidechloride system ($MPO/H_2O_2/Cl^-$ system) was recorded by luminescence after the addition of 50 μM L-012. The HOCl and L-012 interaction derived chemiluminescence was measured in intact MPs using HBSS plus 10 mM HEPES (pH 7.3) and 20 μM H_2O_2. To confirm the luminescence dependency upon chloride, samples of MPs and L-012 were also prepared with HBSS without chlorine. Previously to analyses, baseline readings were performed, and chemiluminescence emission did not exceed 17 counts/s.

For the collected blood smears, stains were prepared using Wright's stain and photographed using optical microscopy. From the same sample, erythrocytes were obtained by centrifugation at $290 \times g$, fixed with half-strength Karnovsky's fixative and examined using a JEOL JSM 6390LV SEM (JEOL Ltd, Tokyo, Japan) electron microscope. The production of ROS by neutrophils and macrophages was assessed when the cells were challenged with freshly washed erythrocytes from patients and controls.

Visualization of erythrocytes derived from patients and healthy individuals

Erythrocytes were obtained from 2 mL of blood (containing heparin) from septic patients or healthy volunteers. During collection, a drop of native blood was used for preparation of smears (stained with Wright's stain) and visualization of red blood cells by light microscopy. The blood samples were centrifuged at $290 \times g$ for separation of plasma and erythrocytes. The plasma was discarded, and the erythrocytes were washed by centrifugation under the same conditions.

Samples containing 10^6 erythrocytes/mL were left still for 30 min, followed by fixation in Karnovsky's fixative