Life Sciences 89 (2011) 773–778

Contents lists available at SciVerse ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Reactive oxygen and nitrogen species modulate the *ex-vivo* effects of LPS on platelet adhesion to fibrinogen

André Luis Casarin, Maria Elisa Lopes-Pires, Rafael Prada Morganti, Edson Antunes, Sisi Marcondes *

Department of Pharmacology, Faculty of Medical Sciences, University of Campinas (UNICAMP), Campinas (SP), Brazil

ARTICLE INFO

Article history:
Received 1 March 2011
Accepted 27 August 2011

Keywords:
Platelet adhesion
Lipopolysaccharide
Nitric oxide
Oxidative stress

ABSTRACT

Aims: Excessive production of nitric oxide (NO) and reactive oxygen species (ROS) in sepsis modulates different cell functions. Since the sepsis severity is associated with the degree of platelet activation, we decided to investigate the role of systemic generation of NO and ROS in modulating the platelet adhesion of lipopolysaccharide (LPS)-treated rats.

Main methods: Platelet adhesion was evaluated using fibrinogen-coated 96-well microtiter plates. Cyclic GMP levels were measured using enzyme immunoassay kit.

Key findings: Treatment of rats with LPS significantly increased spontaneous platelet adhesion, but reduced the thrombin-activated platelet adhesion when compared with control rats. Chronic treatment of rats with the NO synthase inhibitor L-NAME (20 mg/rat/day, 7 days) prior to LPS injection normalized the increased adhesion in non-activated platelets, but failed to affect the adhesion in thrombin-activated platelets. The cGMP levels were modified neither in non-activated nor in thrombin-activated platelets of LPS-treated rats when compared with control rats. The incubation of non-activated platelets with the O_2^- scavenger PEG-SOD reversed the stimulatory effect of LPS on spontaneous adhesion, but had no effect in stimulated-platelet adhesion of non-treated or LPS-treated groups. Moreover, pretreatment of rats with the antioxidant N-acetylcysteine (NAC; 150 mg/kg) prevented the increase of non-activated platelet adhesion, and significantly reduced the inhibitory effect of LPS on thrombin-stimulated adhesion.

Significance: Our findings suggest that in LPS-treated rats, NO plays an important modulatory role only in non-stimulated platelet adhesion through cGMP-independent mechanisms, while ROS, directly or by affecting the redox state of the animals, modulates both non-activated and thrombin-activated platelet adhesion.

Introduction

Platelet activation has a pivotal role in the primary hemostatic event in the cessation of bleeding in damaged blood vessel and it is the primary thrombotic event to precipitate ischemic complications. The activation process occurs through inside-out and outside-in signaling. Inside-out signaling occurs in response to cellular agonist such as thrombin and outside-in signaling is an integrin-mediated cellular event (Lévi-Toledano, 1999). The αIIbβ3 is an important integrin to mediate outside-in signaling and is involved in both platelet adhesion and aggregation. In unstimulated platelets, the αIIbβ3 integrin is an inactive form, and has low affinity for soluble or immobilized fibrinogen and von Willebrand factor (Savage et al., 1992). When platelets are stimulated by different agonists, the αIIbβ3 is converted from low- to high-affinity receptor. Outside-in αIIbβ3 signaling determines the extent of platelet aggregation and spreading (Shattil, 2005).

In addition to regulate primary hemostasis, previous works indicate that activated platelets contribute to inflammatory processes, including sepsis. Accordingly, the first signals in sepsis are the reduction of leukocyte and platelet number in peripheral blood. In addition, the severity of sepsis correlates to platelet activation state (Russwurm et al., 2002). Lipopolysaccharide (LPS) is a component of the outer membrane of Gram-negative bacteria and has a crucial role in inducing Gram-negative sepsis. LPS interacts with a number of cell types such as endothelial cells, macrophages, leukocytes and platelets, causing the release of different substances including cytokines, chemokines, reactive oxygen (ROS) and nitrogen species (RNS) (McCuskey et al., 1996). These reactive species are important to modulate the cell function, and in combination with endogenous antioxidant systems are crucial to the maintenance of the redox balance in the organism (Valko et al., 2007).

The effects of LPS on platelet activity are quite conflicting. LPS administration to mice or rats increases platelet adhesion to intestinal venules (Katayama et al., 2000; Cerwinka et al., 2003). More recently,

* Corresponding author at: Department of Pharmacology, Faculty of Medical Sciences, University of Campinas (UNICAMP), 13084-971, Campinas (SP), Brazil. Tel.: +55 19 3521 9531; fax: +55 19 3289 2968.
E-mail addresses: sisimp@fcm.unicamp.br, s_marcondes@uol.com.br (S. Marcondes).

0024-3205/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.lfs.2011.09.004

LPS in vitro was shown to increase thrombin- and collagen-induced platelet aggregation, and to stimulate platelet secretion of dense and alpha granule (Zhang et al., 2009). In contrast, LPS in vitro has been shown to inhibit platelet aggregation induced by ionophore A23187 and collagen (Saba et al., 1984; Sheu et al., 1998, 1999). In addition, LPS reduces Ca^{2+} mobilization and thromboxane A_2 formation in platelets, and augments the nitric oxide (NO)/cGMP levels (Sheu et al., 1998, 1999).

As mentioned above, most of the studies have been carried out using LPS in vitro and only few works investigated the in vivo effects of LPS on platelet reactivity (Katayama et al., 2000; Cicala et al., 1997). Data obtained with ex-vivo LPS-treated platelets are of potential interest because it reflects the direct effect of LPS and the substances generated during the progress of sepsis. In the present study our hypothesis was that substances released in sepsis modulate the platelet activity. Therefore, the objective of the present work was to study the effects of intraperitoneal treatment of rats with LPS on ex-vivo platelet adhesion to fibrinogen. The modulatory role of ROS and RNS in the platelet adhesion has also been investigated.

Materials and methods

Materials

Lipopolysaccharide from *Escherichia coli* (type 0111:B4), fibrinogen (fraction I from human plasma), thrombin, N-Acetylcysteine, polyethylene glycol-superoxide dismutase (PEG-SOD), N^{ω}-nitro-L-arginine methyl ester (L-NAME), phosphatase substrate (p-Nitrophenyl phosphate, sodium), 3-Isobutyl-1-methylxanthine (IBMX) were purchased from Sigma Chem. Co. (St. Louis, MO, USA). Cyclic GMP enzyme immunoassay (EIA) kits were purchased from Cayman Co. (Ann Arbor, MI, USA).

Experimental protocol

The present study was approved by the Ethical Principles in Animal Research adopted by the Brazilian College for Animal Experimentation (COBEA). Male Wistar rats (250–320 g) were housed in temperature-controlled rooms and received water and food ad libitum. Rats were injected i.p. with saline (0.3 ml) or LPS (1 mg/kg), and at 2 h to 48 h thereafter blood was collected. In a second experimental group, N-acetylcysteine (NAC) was injected (150 mg/kg, i.p.) 30 min after LPS or saline injection (Victor et al., 2003). Finally, in a third experimental group, rats were chronically treated with the NO synthase inhibitor L-NAME (20 mg/rat/day) in the drinking water for 7 days before LPS injection.

Washed platelet preparation

Rats were anaesthetized with isoflurane and the blood was collected from abdominal aorta in 1:9 (v/v) of ACD-C (12.4 mM sodium citrate, 13 mM citric acid, 11 mM glucose). First, platelet-rich plasma (PRP) was obtained by centrifugation of whole blood at 200 *g* for 15 min at room temperature. Five milliliters of PRP was added to 7 ml of washing buffer (140 mM NaCl, 0.5 mM KCl, 12 mM trisodium citrate, 10 mM glucose, 12.5 mM saccharose, pH6), and centrifuged (800 *g*, 13 min). The pellet was resuspended in washing buffer, and the procedure was repeated once. The platelets were gently suspended in Krebs solution (118 mM NaCl, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.7 mM $MgSO_4$, 5.6 mM glucose, pH 7.4). The platelet number was adjusted to 1.2×10^8 platelets/ml in the presence of 1 mM $CaCl_2$.

Platelet adhesion assay

Adhesion assay was carried out according to previous studies (Bellavite et al., 1994; Marcondes et al., 2006). Briefly, the 96-well microtiter plates were coated (overnight at 4 °C) by adding 50 μl per well of human fibrinogen (50 μg/ml). Before use, the wells were washed twice with Krebs solution. The nonspecific adhesion was blocked by incubation of wells with 1% BSA (1 h, 37 °C). At the end of incubation, the plate was washed again, and platelet suspension (50 μl containing 6×10^6 platelets) was added to each well. Platelet suspension, in the absence or in the presence of thrombin (0.1–50 mU/ml), was allowed to adhere to the wells for 30 min at 37 °C. In some experimental protocols, platelets were pre-incubated with PEG-SOD (30 U/ml, 5 min) prior to thrombin addition. Thereafter, the plates were carefully washed twice with 200 μl/well of the Krebs solution to remove unattached platelets. Adherent platelets were quantified through the measurement of acid phosphatase activity. The wells containing adherent platelets were incubated with 150 μl/well of acid phosphatase substrate solution (0.1 M citrate buffer pH 5.4, containing 5 mM p-nitrophenyl phosphate and 0.1% Triton X-100) and after 1 h of incubation at room temperature the reaction was stopped, and the color was developed by addition of 100 μl of 2 N NaOH. The *p*-nitrophenol produced by the reaction was measured with a microplate reader at 405 nm (Spectra Max 340, Molecular Devices, Sunnyvale, CA). The percentage of adherent cells was calculated on the basis of a standard curve obtained with known numbers of platelets. All the experiments were performed in triplicates.

Tail-cuff pressure measurements

Tail-cuff pressure was evaluated before starting the chronic treatment with L-NAME, and at the end of treatment, prior and after LPS injection.

Cyclic GMP determination

Platelets (1.2×10^8 platelets/ml) were incubated with the phosphodiesterase inhibitor 3-isobutyl-l-methylxanthine (IBMX, 2 mM) for 15 min at room temperature. Next, non-activated or thrombin (30 mU/ml)-activated platelets were incubated with Krebs solution for 30 min. Some of these experiments were carried out in the presence of PEG-SOD (30 U/ml). The reaction was interrupted by the addition of cold acidified absolute ethanol to a final concentration of 67% (v/v), and samples were vigorously agitated for 30 s. Cell samples were then incubated on ice for 30 min before centrifuging at 4000 *g* for 30 min at 4 °C. Supernatants were collected and retained and the precipitates washed with 0.5 ml 67% (v/v) acidified ethanol before centrifuging again at 14,000 *g* for 5 min at room temperature. Supernatants from these washed samples were collected and added to the first supernatants collected and dried at 55–60 °C under a stream of nitrogen in a water bath and stored at −20 °C until measurement of cGMP. Cyclic GMP was measured using a kit from Cayman Chemical Co. (Ann Arbor, MI, USA).

Statistical analysis

Data are expressed as means ± SEM of *N* animals. The statistical significance between groups was determined by using one-way ANOVA followed by the Tukey test. A p value of less than 0.05 was considered statistically significant.

Results

Effect of LPS administration in rats on platelet adhesion to fibrinogen

Significant platelet adhesion (6×10^6 platelets/well) was observed when non-activated platelets were kept on plates for 30 min (5.3 ± 0.2%). The adhesion was significantly increased when platelets were activated with 30 mU/ml of thrombin (50 ± 5%; $p<0.05$).

Fig. 1A shows that adhesion of non-activated platelets was significantly increased at 2, 6 and 8 h after LPS injection when compared