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The role of nitric oxide in the epigenetic regulation of THP-1 induced by lipopolysaccharide

Ester Correia Sarmento Rios [a,*], Thais Martins de Lima [a], Ana Iochabel Soares Moretti [b], Francisco Garcia Soriano [a]

[a] Universidade de São Paulo Medical School, Department of Emergency Medicine, Avenida Doutor Arnaldo, 455, Room 3189, São Paulo, SP CEP 01246903, Brazil
[b] Instituto do Coração, Avenida Doutor Enéas de Carvalho Aguiar, 44-Cerqueira Cesar, São Paulo, SP, Brazil

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ABSTRACT

Aims: Changes in the gene expression are one of the molecular events involved in the Systemic of Inflammatory Response Syndrome during sepsis. The preconditioning with low doses of lipopolysaccharide (LPS) reduces the expression of pro-inflammatory genes leading to less tissue damage and better outcome. This hyporesponsive state called tolerance is associated to alterations in chromatin structure and nitric oxide (NO) production. In the current study, we demonstrated that tolerance induced by LPS was found to be NO-dependent and related to epigenetic changes.
Main methods: THP-1 cells were cultivated in RPMI medium (Control), submitted to tolerance (500 ng/mL of LPS 24 h before challenge with 1000 ng/mL of LPS during 24 h Tolerant group) and challenge (1000 ng/mL of LPS during 24 h Directly challenged group). The analyses performed were: cytokines production, histone acetyl transferases/histone deacetylases (HAT/HDAC) activity, nitrosylation of HDAC-2 and -3, expression of acetylated histones H3 and H4. HDAC and Nitric Oxide Synthases (NOS) activities were inhibited with 30 mM trichostatin (TSA) and 100 μM LNAME, respectively.
Key findings: Administration of low doses of LPS repressed the production of IL-6 and IL-10, however this effect was abolished with the inhibition of NOS activity and by TSA in the case of IL-10. Tolerance modulates the activity of HAT and, consequently, the acetylation of histones H3 and H4. Inhibition of NO decreases acetylation of Histones. The HDACs 2 and 3 were nitrosylated after the tolerance induction.
Significance: The tolerance to LPS regulates the cytokine production by modulating chromatin structure and this event is NO dependent.

1. Introduction

Lipopolysaccharide (LPS) has an important role in the systemic inflammation resulted from infection. LPS is the main component of the membrane of Gram-negative bacteria and is recognized by Toll-like receptor (TLR) 4 by monocytes and macrophages during the innate immune response [1]. TLR4 activates several signaling cascades, including the MAP kinases, NFκB, and IRFs leading to the expression and release of several inflammatory mediators in order to protect host from infection [1]. On the other hand, deregulated inflammatory response leads to tissue pathology and underlies many human diseases [2,3]. Systemic Inflammatory Response Syndrome (SIRS) associated to sepsis is defined as a deregulated response characterized by molecular events such as alteration in the gene expression with excessive release of inflammatory mediators [4]. Sepsis and its complications remains a challenge in the intensive care units, presenting high morbid and mortality until nowadays [2].

Despite the role of LPS in sepsis and SIRS, it has been known that the previous stimulus with low doses of LPS can reduce the intensity of the inflammatory cascade. LPS tolerance induces an acquired state of regulated inflammatory response and transcription alterations before the acute phase of immune response [5,6]. This pre-conditioning protects different tissues against subsequent injuries in experimental models [7].

Multiple mechanisms have been proposed to explain *in vitro* LPS tolerance [8,9]. For instance negative regulators of TLR4 signaling cause decreased NF-κB and MAP kinase activation in tolerant macrophages [9]. However, LPS tolerance is a complex phenomenon that leads to several changes in the gene transcription once it reduces the expression of several inflammatory genes.

Modifications on chromatin structure are responsible for gene-specific control of transcription [10,11]. Chromatin remodeling, such as histone acetylation and methylation, has been demonstrated after an initial exposure to low LPS doses and it can be the responsible for the change observed in the inflammatory profile [9].

* Corresponding author.
E-mail address: estercsrios@yahoo.com.br (E.C.S. Rios).

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The chromatin modifying enzymes have to be controlled in order to produce the gene specific regulation. We hypothesized that chromatin remodeling in LPS tolerance is a reprogramming mechanism dependent of nitric oxide (NO). NO is a gaseous molecule capable of a covalent incorporation into thiol groups of cysteines by means of cysteine nitrosylation (S-nitrosylation) [11]. S-nitrosylation is a form of post-translational protein modification with similarities to phosphorylation [12]. S-nitrosylation meets the criteria for validation as a signaling mechanism since it is stimulus evoked [13], precisely targeted [14], reversible [15], spatiotemporally restricted [16,17] and necessary for specific cell responses [18]. A vast number of proteins have been shown to undergo stimulus-dependent S-nitrosylation, both in the nucleus [19–21] and in the cytoplasm [19]. Nitric oxide regulates some proteins, enzymes and transcription factors involved in the immune response [11,22,23]. Different studies also addressed the role of Nitric Oxide (NO) in the interaction between chromatin and transcription factors [22,23]. NO is capable to modulate the chromatin state by post-translational modifications of HDAC enzymes [11]. We investigated the role of NO in the epigenetic regulation mediated by previous doses of LPS.

2. Methods

2.1. Cell culture

THP1 cells were maintained in RPMI 1640 supplemented with 2 mm l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FBS (Sigma). Ultrapure *Escherichia coli* 0111:B4 LPS free of lipoproteins was obtained from Invitrogen (San Diego, CA).

THP1 cells were plated in 60-mm tissue culture dishes (5×10^6 cells/dish). THP-1 were preincubated with 500 ng/ml LPS (TD — tolerant) in 1% FSB RPMI 1640 during 24 h before the challenged with 1 μg/mL LPS during additional 24 h. The Directly challenged group (D) was stimulated directly with LPS 1 μg/mL without the pre stimulus with low dose. Cell culture of Control group (C) was maintained in 1% FBS RPMI 1640. For blocking experiments, the cells were incubated with 100 μM of L-NAME or 30 nM trichostatin (TSA) 30 min before the tolerance or challenge. The LPS dose used to induction of the tolerance as well as to the challenge does not change cell viability in this model [6,9].

2.2. Immunoblotting

Cells were submitted to lyses in a buffer containing 20 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100 and proteolytic enzyme inhibitors (40 μg/ml of phenylmethylsulfonyl fluoride and 10 μg/ml of pepstatin; Sigma). The supernatants were preserved and protein concentration was determined by the Bradford method (Bradford protein assay kit; Bio-Rad, Hercules, CA). The samples were stored at −80 °C until assayed. Protein expression was determined by electrophoresis on a sodium dodecyl sulfate (SDS)-polyacrylamide gel under reducing conditions. Cell extracts (25–100 μg/ml) were boiled in equal volumes of loading buffer (150 mM Tris–HCl, pH 6.8; 4% SDS; 20% glycerol; 15% β-mercaptoethanol; and 0.01% bromophenol blue) and were electrophoresed on 10% polyacrylamide gels. After electrophoretic separation, proteins were transferred to Hybond-P membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK) for Western blotting. Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline and 0.5% Tween 20 (TBST) for 1 h. We employed the following primary antibodies: rabbit acetylated Histone H3 at N-terminal tail (Millipore 06-599), anti-Histone H4 acetyl lys 5/8/12/16 (Millipore) and β-actin (Abcam 16039). The primary antibodies were incubated overnight at 4 °C and the membrane was washed twice in TBST. A secondary horseradish-peroxidase-conjugated antibody (goat anti-rabbit Invitrogen) was then applied at a dilution of 1:5000 for 2 h. Over a 30-min period, the blots were washed twice in TBST, after which they were incubated in enhanced chemiluminescence reagents (Super Signal Detection Kit; Pierce, Rockford, IL, USA). The band intensity of original blots was quantified using Genetools (Syngene, Synoptics Ltd., USA) and was normalized to β-actin expression.

2.3. S-nitrosylation by biotin-switch assay

The plates were washed in phosphate saline buffer with Neocuproine (0.1 mM) and EDTA (1 mM). Cells were lysed in Biotin lysis buffer (250 mM HEPES, 1 mM EDTA and 100 mM neocuproine, 5% CHAPS, 1% SDS, NEM20mM). Samples were homogenized for 1 h — 4 °C in the dark. Proteins were acetone precipitated at −20 °C and then submitted to blocking buffer (HEN buffer plus 2.5% SDS, HENS) with 20 mM methyl methane thiosulfonate (MMTS). After acetone precipitation, nitrosothiols were then reduced to thiols with 1 mM ascorbate. The biotinylated proteins were precipitade with Streptavidin-agarose beads. The supernatant was collected to perform a control western blot of the enzymes of interest. Immunoblotting analysis of proteins collected from beads was performed to detect the amount of nitrosylated HDAC2 and 3 corrected by the amount from supernatant. We employed the following antibodies: rabbit anti-HDAC2 (Santa Cruz Biotechnology sc-7899), rabbit ant HDAC3 (sc-11417).

2.4. Cytokine production

Medium were collected to cytokines IL-6 and IL-10 measurement by ELISA according to manufacturer instructions (R&D Technologies, USA).

2.5. HDAC/HAT activity

The activity of enzymes involved in the epigenetic regulation was analyzed in the cell extract according to manufacturer instructions. HDAC activity was analyzed by colorimetry with Biovision HDAC Activity Assay Kit (K331-100). The activity of HAT was analyzed using Histone Acetyltransferase Activity Assay (Abcam 65352).

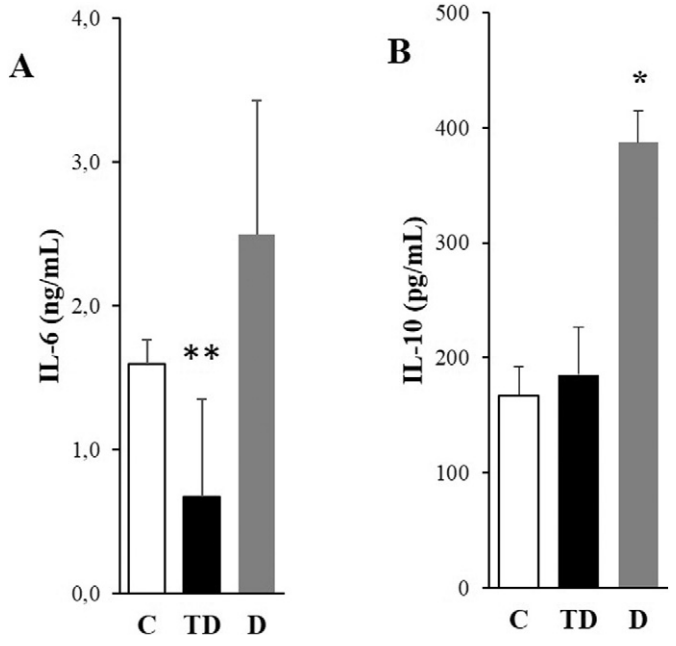

Fig. 1. IL-6 and IL-10 released by THP-1 cells stimulated with single LPS challenge (D) or tolerance (TD). LPS challenge increased IL-6 (A) and IL-10 (B) production by THP-1. However the induction of the tolerance reduced the production of both cytokines. $p < 0.05$ *vs.* Control (C) and Directly challenged (D) groups; $^{**}p < 0.05$ *vs.* D.