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Hemolysin from *Escherichia coli* induces oxidative stress in blood

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ABSTRACT

Hemolysin (HlyA) produced by some stains of *Escherichia coli* is considered to be an important virulence factor of those bacteria. On the other hand, reactive oxygen species (ROS) have been reported to be involved in the pathogenesis of different diseases via oxidative stress generation. The purpose of this study was to analyze the capacity of HlyA to induce oxidative stress in whole blood cultures (WBCs). To this end, ROS production, the damage induced in lipids and proteins, and the antioxidant defense system was evaluated in blood cultures exposed to low concentrations of HlyA. We found that HlyA increased the level of free radicals detected by chemiluminescence assay. Moreover, lipid peroxidation and protein damage was significantly increased in cultures treated with HlyA in comparation with those found in control cultures. On the other hand, a decrease in total antioxidant capacity of plasma and in the activity of superoxide dismutase (SOD) was observed in plasma from blood treated with HlyA. Collectively, our data demonstrate that low concentrations of *E. coli* hemolysin induced oxidative stress in WBCs with the induction of different oxidative damage biomarkers.

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1. Introduction

Escherichia coli is an important food-borne pathogen in Argentina and other parts of the world. The infections caused by these bacteria are responsible for widespread disease, including for example, hemolytic uremic syndrome (HUS), pyelonephritis, septicemia and gastroenteritis (Heffernan et al., 2009; Bentancor et al., 2007). During the infection, *E. coli* secretes different products, including shiga toxin (Stx) and Hemolysin (HlyA) (Proulx et al., 2001; Welch et al., 1995) with the latter being a pore-forming toxin which requires the *hlyCABD* operon for its correct synthesis and extracellular liberation (Welch et al., 1995). Numerous effects on different cellular populations have been attributed to sublytic concentrations of HlyA, including lipoxygenase product formation, liberation of reactive oxygen species (ROS) such as the superoxide anion (O_2^-), and also of reactive nitrogen intermediates (RNI), for example nitric oxide (NO) (Grimminger et al., 1991; Bhakdi and Martin, 1991; Suttorp et al., 1993).

Oxidative stress is caused by an imbalance between the production of oxidants, such as the free radicals, peroxide and nitric oxide, and the levels of antioxidants present in the biological system. In this situation, the overproduction of ROS can lead to the damage of cellular components including lipids, protein, and DNA (Albesa et al., 2004; Baronetti et al., 2011). If this damage is not repaired, mutagenesis and cellular death can occur, which probably participate in the pathogenesis of different diseases such as Alzheimers, multiple sclerosis, diabetes and infectious diseases including Chagas disease, bacterial meningitis and

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hepatitis (Moreira et al., 2005; Gonsette, 2008; Hakim and Pflueger, 2010; Gupta et al., 2009; Kastenbauer et al., 2002; Pal et al., 2010).

Many different biomarkers have been used to follow the development of the oxidative stress in these diseases. For example, carbonyl residues and advanced oxidation protein products (AOPP) are the two parameters usually used to evaluate the level of oxidative damage to proteins (Fredriksson et al., 2005; Halliwell and Whiteman, 2004). In addition, with respect to the lipidic damage induced by oxidating stress, malonyldialdehyde (MDA) is also a well-known biomarker of this process (Niki, 2009). On the other hand, this oxidative imbalance, with over-production of ROS, can be caused by a reduction in the oxidative defenses which are insufficient to removal the free radicals and therefore the antioxidant system plays a very important role in the control of this process. The main enzymes of this system, involved in the detoxification of ROS, are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GSSGR) (Sardesai, 1995; Inal et al., 2002). With respect to SOD, which reduces O_2^- concentrations, a decrease in its activity has been observed in diseases associated with oxidative stress, such as chronic bacterial prostatitis and schizophrenia (Lou et al., 2006; Zhang et al., 2006).

In conclusion, considering that oxidative stress has been linked to illnesses associated with *E. coli* infections such as HUS and pyelonephritis (Celik et al., 2007), and that HlyA is a very important factor of virulence of this bacterium, the objective of this study was to analyze the capacity of this toxin to induce oxidative stress in whole blood cultures (WBCs), which could contribute to the understanding of the pathogenesis of infection by this pathogen.

2. Material and methods

2.1. Culture conditions and purification of E. coli HlyA

The clinically isolated *E. coli* (associated with hemolytic uremic syndrome) was kindly provided by the Microbiology Laboratory of the Pediatric Hospital of Córdoba, Provincia de Córdoba, Argentina. Stock cultures were preserved at −80 °C using glycerol 1% (v/v) as the cryoprotectant and the *E. coli* strain was grown in tryptic soy broth at 37 °C. Bacteria were then pelleted by centrifugation at 16,000 *g* for 15 min at 4 °C, and the cell-free culture supernatants were obtained. The supernatant was precipitated with solid ammonium sulfate (55% w/v) at 4 °C and after 1 h the precipitate was collected by centrifugation (20,000 *g*, 30 min, 4 °C), redissolved in TCU buffer (20 mM Tris/HCL, 150 mM NaCl, 6 M urea; pH 7.0) and dialyzed. Then, the sample was subjected to gel chromatography in a Sephadex G-200 (40 cm long and 2 cm in diameter) and eluted with TCU buffer. The fractions obtained were assayed for proteins and for hemolytic activity and the toxin purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with silver staining (Paraje et al., 2005a, b).

2.2. Hemolytic activity

Erythrocytes were obtained from the heparinized blood of healthy individuals after University Human Ethics Committee protocol approval. Red cells were washed three times with phosphate-buffered saline (PBS)-albumin (0.1 mg/ml) plus $CaCl_2$ (20 mM) before suspension preparation. To 100 μl of diluted toxin, 100 μl of a 0.6% suspension of washed erythrocytes were added which was followed by incubation at 37 °C for 30 min. The percentage of hemolysis was determined by measuring the absorbance at 540 nm, and 100% of lysis was reached by incubation of erythrocytes with water. The hemolytic activity (hemolitic activity (HU/ml)) was taken to be the dilution of HlyA preparation able to produce 50% lysis in a 0.6% erythrocyte suspension (Paraje et al., 2005a, 2005b).

2.3. Detection of ROS by chemiluminescence (CL)

The capability of HlyA to generate ROS was examined in venous whole blood cultures (WBCs) using 3-aminophthalhydrazide 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol) sensitized chemiluminescence (CL). All experiments were carried out following the guidelines of the Ethics Committee of Cordoba University with blood of normal volunteers. The production of ROS in 10 μl of whole blood was measured with 10 μl of Hanks'Balanced Salt Solution (HBSS) and 600 μl of reagent mixture, composed of 5 ml of 0.067% luminol in HBSS, 0.2 ml of 5% glucose, 1 ml of Ringer Lactate Solution and 3.6 ml of distilled water. Then, 10 μl of different concentrations of toxin were added and the CL was tested in a BioOrbit luminometer with the light emission results being expressed as relative light units (RLU). Controls were performed with 10 μl of PBS instead of toxin (Paraje et al., 2005a, 2005b).

2.4. Culture conditions

Two ml of whole blood were incubated with an equal volume of HlyA (0.4 and 0.2 HU/ml) or PBS negative control for 4 h. Then, the blood samples were centrifuged at 900 *g* for 10 min, and the plasma obtained was transferred into separate tubes.

2.5. Lipid peroxidation

150 μl of plasma sample was mixed with 300 μl of a TCA–TBA–HCl reagent [Trichloroacetic acid (TCA): 15% w/v, thiobarbituric acid (TBA) 0.375%, hydrochloric acid (HCl) 0.25 N] and heated in boiling water for 30 min. An ice bath was then used to cool the samples, after which, they were centrifuged at 1500 g and the absorbance of the supernatant was determined by spectrophotometry at 535 nm. The reference standard used was 1,1,3,3 tetraethoxypropane and MDA levels were expressed in μmol/L (Becerra et al., 2006).

2.6. Determination of advanced oxidation protein products (AOPP)

One ml of plasma diluted 1/5 in PBS was analyzed with 0.1 ml of acetic acid and 50 μl of potassium iodide (IK)

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