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Research Article

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Subinhibitory concentrations of amoxicillin on *Helicobacter pylori* increase apoptosis in RAW 264.7 cells

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ABSTRACT

Subinhibitory concentrations of antibiotics have been shown to alter the virulence of microorganisms, which may modify the progression of infection. This study assessed the induction of nitric oxide and apoptosis on macrophages infected by Helicobacter pylori exposed to subinhibitory concentrations of amoxicillin. RAW 264.7 (macrophage-like cells) infected with H. pylori amoxicilin-treated significantly increased the levels of nitric oxide and apoptosis ininfected macrophages as compared to H. pylori without treatment. These results suggest that amoxicillin at subinhibitory concentrations can contribute to stimulate immune response in the host by inducing over-expression of some specific inflammatory mediators.

Key words: *Helicobacter pylori*, subinhibitory concentration, amoxicillin, nitric oxide, apoptosis.

INTRODUCTION

Helicobacter pylori is a prevalent Gram-negative microaerophilic bacterium that colonizes the mucus layer in the stomach and produces various virulence-associated factors that have been extensively characterized [1]. The presence of *H. pylori* and development of inflammatory response depends on interaction between the bacterium and the host[2]. The infection caused by *H. pylori* results in a large influx of immune cells including neutrophils, macrophages, dendritic cells, lymphocytes, and an associated innate and adaptive immune response [3].Nitric oxide (NO) is an important effector possessing antimicrobial activity and immunomodulatory effect.NO and reactive nitrogen intermediates (RNI) play protective roles in the acute and persistent phases of *H. pylori* infection [4,5].When NO diffuses into bacterial cytoplasm, peroxynitriteis formed via interaction with O_2. Subsequently, peroxynitrite can oxidate, S-nitrosatemicrobial proteins, nucleic acids and lipids, exerting toxic effect. On the other hand, NO and RNI can react with thiols to form S-nitroso compounds, therefore cell respiration and metabolism of *H. pylori* can be inhibited [5,2].

The mechanism by which *H. pylori* promotes apoptosis on human cells has not been completely understood. Although excessive NO generation and a massive rise in apoptosis are well recognized, cytokines (TNF-α, interferon-gamma (IFN-γ), IL-2 and IL-1), ascorbate, and ammonia also activate apoptosis [6]. The formation of intracellular reactive nitrogen species, with increased level of nitric oxide synthase (iNOS) [7]can cause cell DNA damage, mutagenesis, apoptosis and development of gastric carcinoma [4,5,8].

The most commonly used regimens for the eradication therapy of *H. pylori* consist of a combination of two antibiotics (usually metronidazole, clarithromycin, amoxicillin, tetracycline or furazolidone) associated with a proton pump inhibitor (omeprazole, lansoprazole or pantoprazole) [9]. The efficacy of this therapy depends on

antibiotic drug resistance, patient compliance and drug-related side effects [10]. Several studies have demonstrated that the antimicrobial inhibitory concentration (MIC) [11] or subinhibitory concentrations (sub-MIC) might alter the bacterial virulence factors, and as result the relationship between microorganism and host [12].

Considering that the eradicating therapy of *H. pylori* is not always effective and that amoxicillin is included in treatment regimens, the purpose of the present study was to assess whether levels of NO release by macrophages and apoptosis are altered if cells from *H. pylori* grown in the presence or absence of subinhibitory concentrations of amoxicillin are used as stimulus.

EXPERIMENTAL SECTION

Helicobacter pylori strain, amoxicillin susceptibility and growth conditions

H. pylori ATCC 43504, metronidazole resistant and amoxicillin susceptible, was cultured in Columbia agar containing 10% fetal bovine serum (FBS) and incubated at 37°C for 72 hours, in 5% CO_2. The minimal inhibitory concentration (MIC) of amoxicillin (Lot number: 080616478, Pharma Nostra, Rio de Janeiro, Brazil) was determined using the broth microdilution method as previously described [13]. The wells of a 96-well microplate were filled with 100 µl of various concentrations of amoxicillin. Same volume of *H. pylori* suspension (about 10^6 CFU/ml) was added to each well. The absorbance was determined using an automatic ELISA microplate reader (Spectra & Rainbow Readers, Tecan) adjusted at 620 nm. The microplate was incubated at 36–37°C for 3 days, under microaerophilic atmosphere, agitated and the absorbance was read again in the reader at the same wavelength. The absorbencies were compared to the values obtained before incubation to detect an increase in bacterial growth. The lowest concentration of the test amoxicillin resulting in inhibition of bacterial growth and the obtained MIC value was 0.250µg/ml. *H. pylori* was cultured in Brain Heart Infusion supplemented with 10% FBS in the presence or absence (HP) of amoxicillin at sub-MICs (½ and ¼ MIC) at 37ºC under microaerophilic atmosphere for 24 hours. In the nitric oxide assay, bacterial cells were washed twice with sterile phosphate buffered saline (0.05 mol/l and pH 7.2), centrifuged at 6000×*g* for 10 min, and suspended in Dulbecco's modified Eagle's medium in standard inoculum corresponding to 10^9 CFU/ml (UDO620 0.40).

RAW 264.7 macrophage-like cells stimulation and NO production

RAW 264.7 macrophage-like cells (American Type Culture Collection ATCC, TIB 71, Rockville, MD) were cultured in Dulbecco's modified Eagle's medium containing L - glutamine and glucose (Gibco BRL Life Technologies) supplemented with 10% FBS and 10 µg/ml gentamicin (Gibco BRL Life Technologies) in air with CO_2 5% at 37°C. The cells (1×10^6 cells/500 µl/well) in the presence or absence (HP) of amoxicillin at sub-MICs (½ and ¼ MIC) (500 µl as described) were incubated for 24 hours at the same conditions. Each experiment was accompanied by controls (10 µg/ml lipopolysaccharide, LPS, Sigma, and Dulbecco's modified Eagle's medium). After incubation, the cells were washed three times with phosphate buffered saline (0.05 M and pH 7.2) and infections were done with *H. pylori* as described. The plates were incubated for 24 hours. After incubation, the supernatants were collected. The levels of nitric oxide were determined by measuring the amount of nitrite, a stable metabolic product of nitric oxide, as previously reported [14]. The assay mixture contained medium (50 µl) plus Griess reagent (50 µl, Merck), and absorption was measured at 540 nm using a microplate reader (Spectra & Rainbow Readers, Tecan). The results were reported as the amount of nitrite (µmol/ml). Experiments were carried out in triplicate and repeated at least twice. The results were expressed through mean ± sd. Statistical comparisons were performed using Student's t test data ($p < 0.05$).

Assessment of apoptosis

The binding of annexin V - fluorescein isothiocyanate was used as a measurement of apoptotic RAW 264.7 cells with an annexin V - fluorescein isothiocyanate – propidium iodide apoptosis detection kit (BD Biosciences Pharmingen) according to the manufacturer's instructions. Cells were pooled, pelleted by centrifugation, washed once with ice-cold phosphate buffered saline, and resuspended in binding buffer to a concentration of 10^6/ml. Next, 0.1 ml of this cell suspension was transferred to a tube and incubated with 5 µl of annex in V-fluorescein isothiocyanate and 5 µl of propidium iodide for 10 minutes at 25 °C in dark. Finally, 0.4 ml of binding buffer was added, and samples were analyzed by flow cytometry within 1 hour on a FACScan flow cytometer (Becton Dickinson-Bioscience, FACS Calibur TM, San Jose, California, USA), and processed with the integrated software. Uninfected cells without treatment were used as viability control**.** The apoptosis percentage was calculated as follows: apoptosis = number of early and late apoptotic cells/total number of cells × 100 %.Experiments were carried out in triplicate and repeated at least twice. The results were expressed through mean ± sd. Statistical comparisons were performed using Student's t test data ($p < 0.05$).

RESULTS AND DISCUSSION

Induction of nitric oxide (NO) and detection apoptosis in macrophages by H. pylori

Prevalence of *H. pylori* antibiotic resistance is increasing worldwide, and it is has been the main factor affecting efficacy of current therapeutic regimens [8]. Concentrations of antibiotics below the minimum inhibitory are known to induce bacterial virulence that can alter the host response [15].

Inducible iNOS, which produce large amounts of NO, is induced in macrophages in response to inflammatory mediators such as LPS and cytokines [16]. NO production is critical for the host defense against intracellular pathogens during infection, via its antimicrobial and cytoprotective activities[17].In this study, we demonstrated that treatment of HP with 0.063 µg/ml (¼ MIC) of amoxicillin increased of NO and apoptosis (early and late/necrosis) ($p<0.05$) compared to that induced by HP not exposed to antimicrobial agent after 24 hours of incubation (Fig. 1 A and B).A mechanism employed by *H. pylori* to activate iNOS involves urease, an important virulence factor of *H. pylori*. *H. pylori* arginase was shown to be an important factor that affords protection of the bacteria against NO mediated killing since macrophages infected with *H. pylori* produce significantly less NO than arginase isogenic mutants [18]. With appropriate defenses, this oxidative stress would be able to rapidly kill nearby *H. pylori*, but it is known that *H. pylori* combats oxidative stress via diverse activities, some of which are unique. Although NO is an important signaling molecule and a component of inflammatory response, its over production leads to tissue damage and contribute to inflammation [19]. Several reports demonstrated that apoptosis of macrophage can be induced by numerous effectors molecules, including NO, and that modulation of NO production in response to *H. pylori* would regulate levels of macrophage apoptosis [20]. Our results demonstrated a decrease in cell viability and increase macrophage apoptosis (early and late/necrosis) after infection with *H. pylori* previously treated with sub-MICs of amoxicillin. A recent study showed that induction of macrophage arginase II (Arg2) restricts iNOS protein expression, elicits apoptosis of macrophages as well as proinflammatory cytokine production, and limits bacterial killing [21], suggesting another mechanism this bacteria uses to escape macrophage-mediated killing. *H. pylori*Vac A protein also causes apoptosis of monocytes. The underlying mechanism of this process involves the amino-terminal 476 residue fragment (p52) of VacA, which activates the NFκB pathway and induces proinflammatory cytokine production, *e.g.*, TNF-α, IL-1β, and induction of NO, reactive oxygen species and subsequently causes apoptosis of monocytes [22].

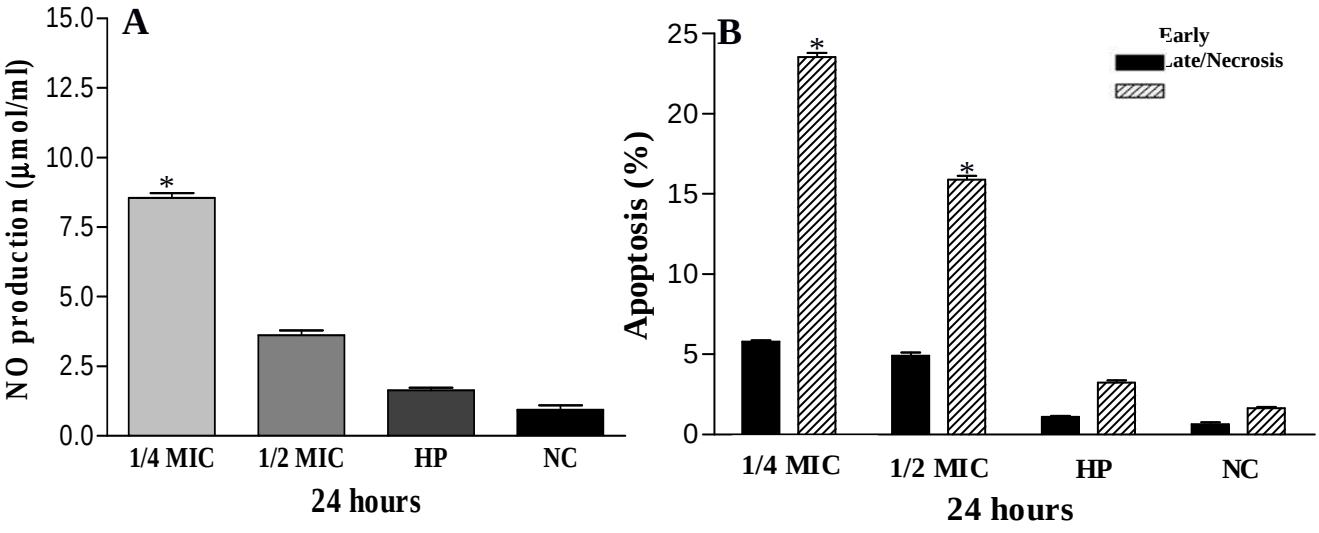

Fig. 1.Inductionof nitric oxide (A) and apoptosis (B) in RAW 264.7 cells treated with *Helicobacter pylori* cultured with amoxicillin at subinhibitory concentrations for 24 hours. A total of 1 × 10^6 cells/500 µl/well was infected with *H. pylori* (10^9 CFU/ml). ¼ MIC: *H. pylori* cultured with amoxicillin at 0.063 µg/ml; ½ MIC: *H. pylori* cultured with amoxicillin at 0.125 µg/ml; HP: untreated *H. pylori*; NC: macrophages (negative control), *: p < 0.001 compared to HP

Bars represents the mean ±SD for at least twice independent experiments carried out in triplicate

Previous *in vitro* studies showed that sub-MICs of beta-lactam antibiotics and fluoroquinolones increase the levels of alpha-toxin expression of *Staphylococcus aureus*, suggesting that the symptoms of *S. aureus* infections may be aggravated by penicillin treatment [15,23,24]. Our results provide evidence that amoxicillin, at sub-MICs, increases the immune response in *H. pylori* infected macrophages by inducing inflammation through specific inflammatory mediators.

H. pylori induces macrophages apoptosis in order to modulate host immune responses and establish chronic infection [4,5,25]. Corroborating evidence, macrophages exposed to *H. pylori* previously treated with sub-MIC of amoxicillin produce NO and increase apoptosis than HP not exposed to the antimicrobial agent.

CONCLUSION

Numerous studies demonstrated that antimicrobial at sub-MICs may alter the bacterial virulence factors and the microorganism-host relationship. This study demonstrate that amoxicillin at subinhibitory concentrations has an effect on the virulence of *H. pylori* that enhances the inflammatory process and behavior of susceptible *H. pylori* that can contribute to the pathogenicity of the bacterium.

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