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TLR2 activation induced by *H. pylori* LPS promotes the differential expression of claudin-4, -6, -7 and -9 via either STAT3 and ERK1/2 in AGS cells

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ABSTRACT

Gastric carcinogenesis has been associated to *H. pylori* virulence factors that induce a chronic inflammation process. Lipopolysaccharides play a role in chronic inflammatory responses via TLR2- and TLR4-dependent signaling pathways. Similarly, cellular invasiveness, metastatic potential and prognosis are usually associated to claudin-4, -6, -7 and -9 expression in gastric carcinogenesis. Therefore, the aim of this study was to determine if *H. pylori* LPS exerts an influence on carcinogenesis-related claudin expression and if it was directly regulated through the TLR2 pathway. Human antrum gastric adenocarcinoma AGS cells exposed or not to *H. pylori* LPS were used. Polyclonal anti-claudin-4, -6, -7 and -9, anti-TLR2, anti-pERK1/2 as well as rabbit monoclonal anti-pNFκB p65 and mouse monoclonal anti-CdX2 were used. ERK1/2 inhibitor UO126 and STAT3 inhibitor Stattic were also used. Western blot, immunofluorescence and confocal experiments were performed in whole cells as well as total protein, nuclear and cell membrane fractions. The results showed that *H. pylori* LPS increased the expression of TLR2 in a time dependent bi-phasic manner (< 12 and > 12 h exposure). Immunofluorescence using AGS monolayers corroborated the double phase TLR2 expression mainly on the cell membrane but a detectable signal was also determined in the cytoplasm of the cells. Activation of NFkB was downstream and depended on TLR2 expression as a statistically significant increase in pNFkB, that followed a pattern highly similar to the TLR2 expression was observed on the cell membrane fraction. The increase in TLR2 expression was accompanied by dramatically increased claudin-4 expression in cultures exposed from 30 m to 8 h to LPS. Increased expression of claudin-6, -7 and -9 also increases in > 12 h LPS exposure times. The increase in claudins expression was also dependent on NFkB activation. The results also showed an increase in pSTAT3 that followed a bi-phasic pattern that began 30 min after stimulation and was compatible with the increase in TLR2 expression. The expression of the claudin-4 related CDX2 transcription factor did not followed the biphasic pattern. The results also showed that claudin-4 expression was STAT3 dependent whereas claudin-6, 7 and 9 expressions was ERK1/2 dependent. Our results suggest that *H. pylori* LPS induces TLR2 expression in the AGS cells, and that the longer the exposure to LPS, the greater the expression of TLR2 in the cell membrane. Consequently the expression of claudin-4, -6, -7 and -9 also increases.

1. Introduction

Gastric cancer is a leading cause of cancer death worldwide (Torre et al., 2015). The development of gastric cancer is a complex process that involves genetic and epigenetic alterations of oncogenes, cell cycle regulators, signaling molecules and infection by the gram-negative bacterium *Helicobacter pylori* (Nagini, 2012). Gastric carcinogenesis has been associated to the effect that *H. pylori* virulence factors exert upon the gastric epithelia (Shimizu et al., 2015) and it has been clearly established that chronic inflammation by resident microbes in the presence or not of *H. pylori* can lead to alterations in immune regulation that might promote gastric carcinogenesis (Wroblewski et al., 2016). Carcinogenesis is usually associated to the loss of tight junction structure (Martin et al., 2011; Soler et al., 1999) whose key elements are the transmembranal proteins called claudins (Gunzel and Yu, 2013). Although there are several differing reports of diverse claudin expression

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in different gastric cancer types (Jun et al., 2014; Wang and Yang, 2015; Lin et al., 2013) there seem to be an undisputed acceptance that claudin-4, -6, -7 and -9 expression is compatible with increased cancer invasiveness, metastatic potential and prognosis (Zavala-Zendejas et al., 2011; Kwon et al., 2011; Ben-David et al., 2013).

It has been established that lipopolysaccharides (LPSs) can modulate the immune response and play a role in chronic inflammatory responses (Fujimoto et al., 2012) via TLR2- and TLR4-dependent signaling pathways (Pachathundikandi et al., 2015). *H. pylori* LPS is less endotoxic than *E. coli* LPS due to severe modifications on its lipid A core but it can trigger a TLR4 expression or antagonize TLR4 activation (Lepper et al., 2005). However, there are reports that prove the agonist function of *H.pylori* LPS upon TLR2, but not on TLR4 (Smith et al., 2003). Despite the controversial role of LPS in TLR activation it has been shown that TLR4-mediated lipid signaling promotes cell survival and cancer development in colonic carcinogenesis (Kuo et al., 2015). Interestingly, the enhanced tumorigenesis associated to TLR2 in hepatocarcinoma depends on the stage of inflammation (Lopes et al., 2016).

Nevertheless, the role of *H. pylori* LPS, a major component of *H. pylori* cell wall, in gastric carcinogenesis and claudin expression has not been extensively evaluated. The aim of this study was to determine if *H. pylori* LPS exerts an influence on carcinogenesis-related claudin expression and if it was directly regulated through the TLR2 pathway. Our results showed that TLR2 expression and probably its density in the cell membrane induce the expression of epithelial-associated claudin-4, -6, -7 and -9 in the human gastric adenocarcinoma cell line AGS.

2. Materials and methods

2.1. Antibodies and inhibitors

Biotin anti-mouse/human CD282 (TLR2, Cat. # 121804), Donkey anti-rabbit IgG-FITC (Cat. # 406403), anti-mouse IgG- FITC (Cat. # 406001), and Streptavidin-FITC (Cat. # 405201) were from BioLegend (San Diego, CA). Mouse anti Goat/Sheep IgG-FITC (Cat. # F489), Streptavidin-HRP (Cat. # RABHRP3), and NFκB inhibitor Bay 11–7085 (Cat. # B5681) were from Sigma-Aldrich (Saint Louis, Missouri). F(ab') 2-Rabbit anti-Goat IgG (H + L)-HRP (Cat. # A24446), Rabbit anti-Goat IgG (H + L)-HRP (Cat. # 81–1620), Goat anti-Rabbit IgG (H + L)-HRP (Cat. # 31460), and Rabbit anti Claudin 7 (Cat. # PA5-23689) were from Thermo Fisher Scientific, Meridian (Road Rockford, IL). Rabbit polyclonal anti-claudin 4 (Cat. # GTX15104), Rabbit polyclonal anti-claudin 6 (Cat. # GTX115183), Rabbit polyclonal anti-claudin 9 (Cat. # GTX77838), and Mouse monoclonal anti-CDX2 (Cat. # GTX49162) were from GeneTex (Irvine, CA). NA^+/K^+-ATPase α1(F-2) (Cat. # sc-514614), Goat polyclonal anti-claudin 6 (Cat. # sc-17669), Goat polyclonal anti-claudin 9 (Cat. # sc-17672), and Donkey anti-mouse IgG-HRP (Cat. # sc-2314) were from Santa Cruz Biotechnology (Dallas, Texas). Mouse monoclonal Nucleoporin p62 (Cat. # 610497) was from Becton, Dickinson (Franklin Lakes, New Jersey). Rabbit monoclonal anti- Phospho-Stat3 (Tyr705)(Cat. # 9145) and anti-Phospho NF-κB p65 (Ser536)(Cat. #3033), Rabbit polyclonal anti-Phospho-p44/42 MAP (ERK1/2)(Thr202/Tyr204) (Cat. # 9101), Rabbit monoclonal anti-ERK1/2 inhibitor UO126 (Cat. # 9903), and STAT3 inhibitor Stattic (Cat. # 2798) were from Tocris Biosciences (Bristol, UK). Anti β-actin was a generous gift from CINVESTAV, IPN and purified H. *pylori* LPS was kindly donated by Dr. Victor Rafael Coria Jimenez and QFB. Maribel Ortiz Herrera, department of bacteriology, Instituto Nacional de Pediatria, México.

2.2. Cell culture

AGS cell line derived from human gastric adenocarcinoma obtained from American Type Culture Collection (Manassas, VA), were seeded in petri *p*-100 and maintained with Dulbecco's modified Eagle's medium (DMEM; Cat. # 31600-034 Life Technologies, Carlsbad, CA), supplemented with 5% fetal bovine serum (Cat. # 10371-029, Biowest, Nuaillé – France), 2 mM sodium pyruvate (100 Mm, Cat. # L0642-100, Biowest, Nuaillé – France), 2 mM L-Glutamine (200 mM, Cat. # X0551-100, Biowest, Nuaillé – France), 100 U/ml/100 ug/ml penicillin/streptomycin (Cat. # L0010-100, Nuaillé – France), cells were incubated under conditions of 37° C and 5% CO_2.

2.3. Preparation of LPS Helicobacter pylori and exposure to AGS cells

Helicobacter pylori (strain J99) LPS was extracted by hot phenol-water method as described previously (Westphal O, Jann K. 1965). LPS concentration was determined by the phenol-sulfuric acid method. AGS cells were treated with 10 ng/ml *LPS H. pylori* for 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h and 48 h.

2.4. Total protein extraction

After exposure to LPS and inhibitors, the monolayers AGS cells were washed two times with PBS and scraped with 1 ml ice-cold lysis buffer (150 mM NaCl/50 mM Tris/EGTA 1 mM/1 mM EDTA/IGEPAL 1%/Sodium deoxycholate 0.1%/0.1% SDS + protease and phosphatases inhibitors). Cell suspensions were sonicated 1 min at 25% amplitude. It was centrifuged for 30 min at 17,000*g* (4 °C). The supernatants were recovered, and the total protein amounts were determined with a Bio-Rad assay (Cat. # 500-0009, Bio-Rad, Hercules, CA, USA).

2.5. Nuclear protein extracts

Cells were washed two times and scraped with PBS. The cell suspension incubated for 20 min on ice with buffer I (10 mM Hepes-KOH pH 7.9, 1.5 mm $MgCl_2$, 10 mM NaCl + protease and phosphatases inhibitors) was centrifuged 5 min at 1000*g* (4 °C), with 1 ml hypotonic buffer (10 mM Hepes-KOH pH 7.9, 1.5 mm $MgCl_2$, 10 mM NaCl + protease and phosphatases inhibitors). The supernatant was removed and the cell pellet is suspended and incubated for 20 min (4 °C) in buffer C (20 mM Hepes-KOH pH 7.9, 1.5 mM $MgCl_2$, 420 mM NaCl, 25% glycerol, 0.2 mM EDTA + protease inhibitors). Immediately centrifuged at 17,000*g* for 5 min (4 °C). The supernatant (nuclear fraction) were recovered, and the total protein amounts were determined with a Bio-Rad assay (Cat. # 500-0009, Bio-Rad, Hercules, CA, USA).

2.6. Membrane protein extracts

Cells were washed two times with ice cold PBS and incubated for 1 h on ice with buffer A (Tris base 20 mM/Destroxa Anhidra 0.25 mM/EGTA 10 mM/2m + Triton X–100 al 1% + protease and phosphatases inhibitors) and were centrifuged at 17,000*g* for 30 min (4 °C). The supernatants (membrane fraction) were recovered, and the total protein amounts were determined with a Bio-Rad assay (Cat. # 500-0009, Bio-Rad, Hercules, CA, USA).

2.7. Western blot

For Western blot analysis 20 μg of protein was resolved on SDS/PAGE and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked with 5% non-fat dry milk in tris-buffered saline (TBS) and incubated with primary antibodies in Tween 20/TBS (TTBS) overnight at 4 °C. Membranes were then washed with TTBS and incubated with secondary antibodies conjugated to horseradish peroxidase. Antibody binding was detected by chemiluminescence.

Total, nuclear and membrane protein extracts (40 μg for TLR2, CLDN 4, 6, 7 and 9 or 50 μg for phosphorylated proteins) were boiled with Laemmli buffer and β- mercaptoethanol (5%) for 5 min resolved on SDS-PAGE gels (12%, 13% y 15%), transferred to nitrocellulose membranes (Cat. # HATF00010, Merck Millipore, Tullagreen, Co, Cork). Membranes were blocked with 5% non-fat dry milk in TBS and