THE LIPOPOLYSACCHARIDE FROM ESPHERICHA COLI O127:B8 INDUCES INFLAMMATION AND MOTILITY DISTURBANCES IN RABBIT ILEUM

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Abstract: The aim of this work was to evaluate the effects of lipopolysaccharide (LPS) from Escherichia coli O127:B8 on the expression of toll-like receptor 4 (TLR4), the histology, and motor function in rabbit ileum. Rabbits were injected intravenously with saline or LPS (100 µg/kg, 2 h). The mRNA expression and localization of TLR4 were determined by reverse transcriptase-PCR and immunofluorescence, respectively. Histological damage induced by LPS was evaluated in sections of ileum stained with haematoxylin and eosin. Contractility studies of ileum were performed in an organ bath. The mRNA expression of TLR4 decreased in the muscular but not in the mucosal layer of rabbits treated with LPS. TLR4 was localised in both the mucosal and muscular layers of rabbit ileum. LPS induced intestinal inflammation and altered the spontaneous contractions and the serotonin-, acetylcholine- and KCl-induced contractions. In conclusion, LPS from E. coli O127:B8 induced a decrease in the mRNA expression of TLR4, an inflammatory response, and changes in the contractility of rabbit ileum.

Key Words: intestine, motility, lipopolysaccharide, toll-like receptor 4, sepsis, rabbit.

INTRODUCTION

Toll-like receptors (TLRs) are members of the pattern recognition receptors (PRRs) superfamily, which detect pathogen-associated molecular patterns (PAMPs), and they are vital for innate immune response (Takeda and Akira, 2004). Gene expression of TLR2, 3, 4, 5, 6, 8, and 10 has been found in the lung, trachea, intestine, stomach, liver, spleen, utero, ovary, and hypothalamus of control rabbits (Chen et al., 2014). In this context, the lipopolysaccharide (LPS) of Escherichia coli bacteria is recognised by the host intestine through toll-like receptor 4 (TLR4) (Takeda and Akira, 2004).

Intravenous administration of the LPS from E. coli has been widely used as a model of sepsis in several animal species, including monkeys (Imaeda et al., 2002), pigs (Tadros et al., 2000), mice (Arribas et al., 2009; Lyu et al., 2015), rats (Wafa et al., 2015; Li et al., 2016) and rabbits (Gonzalo et al., 2010, 2015). LPS has been reported to evoke an inflammatory response (Lyu et al., 2015) and decrease the blood flow to the intestine, resulting in ischemic damage in the intestinal mucosa and increased epithelial barrier permeability (Tadros et al., 2000; Wafa et al., 2015; Li et al., 2016). In addition, LPS inhibits gastrointestinal motility and induces the release of reactive oxygen species (Hamano et al., 2007; Gonzalo et al., 2010).

Previous studies have revealed that the LPS from E. coli 0111:B4 inhibits the motility and contractility of rabbit small intestine by activating the TLR4 (Gonzalo et al., 2015); the p38, ERK, and JNK Mitogen-Activated Protein Kinases (MAPK) pathways (Gonzalo et al., 2010, 2011a, 2011b); the IkappaB kinase complex and proteasome...
Grasa et al. (Gonzalo et al., 2015); and, finally, inducing the translocation of nuclear factor kappaB to the nucleus (Hernandez et al., 2011) and evoking the release of interleukins (Hernandez et al., 2011; Gonzalo et al., 2015).

Enteropathogenic E. coli 0127:B8 is associated with acute diarrhoea in infants (Cooper et al., 1955), but it has also been reported to be able to attach to rabbit enterocytes (Germani et al., 1985). However, most of the effects of this strain on rabbit intestine remain unexplored. Therefore, the aim of this work was to evaluate the effects of LPS from E. coli 0127:B8 on the expression of TLR4, the histology and motor function in rabbit ileum.

MATERIALS AND METHODS

Animals and treatments

All procedures were carried out under Project Licence 10/11 and approved by the in-house Ethics Committee for Animal Experiments from the University of Zaragoza. The rabbits (male New Zealand, 2–2.5 kg) were injected intravenously (IV) with either saline or LPS from E. coli 0127:B8 (100 µg/kg in saline). The LPS was purchased from Sigma (Madrid, Spain) and according to the data sheet, the LPS was phenol extracted from E. coli serotype O127:B8, strain ATCC 12740. The rabbits were humanely sacrificed by a blow to the head 2 h after the saline or LPS injections. Rectal temperature was measured every 30 min after the saline or LPS treatments.

Reverse transcriptase-PCR

The total RNA from muscular or mucosal tissues of ileum from saline- and LPS-treated rabbits (n=3) was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and the cDNA was synthesised using the AffinityScript Multiple Temperature cDNA Synthesis Kit (Stratagene, La Jolla, CA, U.S.A.). One-tenth of the resulting cDNA was used for the PCR amplification of rabbit genes. Primer sequences were: GAPDH-FW 5’-TCACCATCTTCCAGGAGCA-3’; GAPDH-REV 5’-CACAATGCCGAAGTGGTCGT-3’; TLR4-FW 5’-GAGCACCTGGACCTTTCAAATAAC-3’; and TLR4-REV 5’-GAACTTCTAAACCACTCAGCCCTTG-3’. Polymerase chain reaction (PCR) amplification was carried out after various cycles (22 for GAPDH and 32 for TLR4) consisting of 2 min of denaturation at 94°C, 30 s of annealing temperature (60°C for GAPDH and 57°C for TLR4), and 30 s of extension at 72°C. These conditions were determined after screening (20 to 44 cycles), and the final PCR amplification conditions were chosen so that any of the PCR products analysed reached a plateau at the end of the amplification protocol. PCR products were analysed on 2% agarose gels, and bands were visualised by ethidium bromide staining. The images from the gels were captured with the Biodoc-It Imaging System (UVP Inc., Upland, CA, U.S.A.). The TLR4/GAPDH ratio, in densitometric units, was calculated using the Quantity One software (Biorad Laboratories, Hercules, CA, U.S.A.).

Immunofluorescence

The localisation of TLR4 by immunofluorescence was studied in rabbit ileum fixed in 4% paraformaldehyde for 2 h at RT. The tissue was mounted in Tissue Tek OCT compound (Electron Microscopy Sciences, Hatfield, PA, UK), frozen in dry ice/isopentane and stored at –80°C. After blocking with 2% bovine serum albumin for 30 min at RT, sections were incubated with a mouse monoclonal antibody against TLR4/CD 284 1:50 (MBL International Corporation, Way Woburn, MA, U.S.A) for 45 min at RT and goat antimouse Alexa Fluor 488 1:500 (Invitrogen, Carlsbad, CA, U.S.A.) for 30 min at RT.

Histology

Specimens of ileum were fixed in 10% neutral buffered formalin, dehydrated, and paraffin embedded. Sections were stained with haematoxylin and eosin, and histological damage was evaluated by a person unaware of the treatments.

Motility studies

Contractility studies of whole segments and muscle strips of ileum were performed in an organ bath, as previously described (Gonzalo et al., 2010). Whole segments and muscle strips were suspended in the direction of longitudinal
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Smooth muscle fibres with an initial tension of 2 or 0.5 g, respectively. Serotonin (100 µM), acetylcholine (100 µM), or KCl (80 mM) was added to the bath for 3 min. The amplitude (millinewton, mN) and frequency (contractions per min, cpm) of the spontaneous contractions were analysed. The motor responses to the different agonists were measured as the area under the curve (AUC) and expressed as mN/s per g of intestinal tissue.

**Data analysis and statistics**

Results were expressed as mean ± standard error of mean. Data were analysed by unpaired Student’s t-test, and the differences with $P<0.05$ were considered statistically significant.

**RESULTS AND DISCUSSION**

**Expression and localisation of TLR4 in rabbit ileum**

The treatment with LPS from *E. coli* O127:B8 (100 µg/kg, 2 h, IV) decreased the mRNA expression of TLR4 in the muscular but not in the mucosal layer of rabbit ileum (Figures 1A, B). Our results agree with other authors who have shown that treatment with LPS from *E. coli* O55:B5 (50 µg/kg, 3 h, intra-peritoneal) down-regulates the adrenal mRNA expression of TLR4 in rats (Sanchez-Lemus et al., 2008). On the contrary, LPS from *E. coli* O55:B5 (500 µg/kg, 3 h, IV) has been reported to evoke an increase in the mRNA expression of TLR4 in rabbit ovary and uterus (Chen et al., 2014). Therefore, the effect of LPS on the regulation of TLR4 expression seems to be different depending on the strain of *E. coli* and/or the organ evaluated.

Our immunofluorescence experiments revealed the presence of TLR4 in both the mucosal and the muscular layers of rabbits treated with LPS (Figures 1C, D). In the mucosa, the signal was stronger in the apical membrane of the epithelial cells (Figure 1C). Previously, TLR4 expression has been reported in the apical membrane of the ileal epithelial cells in both control and intraluminally injected-with-LPS monkeys (Imaeda et al., 2002).

**Effects of LPS from *E. coli* O127:B8 on rectal temperature and histology of ileum**

The treatment with LPS induced a time-independent increase in the rectal temperature of the rabbits (saline: 39.7±0.1, 39.2±0.1, 39.2±0.2, 39±0.2, 39.1±0.2; LPS: 39.6±0.2, 40.2±0.2, 40.3±0.1, 40.5±0.1, 40.6±0.2°C, at 0, 30, 60, and 90 min after the treatment, respectively; $P<0.01$; n=7), in agreement with the results obtained by other authors using rats (Dogan et al., 2000). The ileum from rabbits treated with LPS showed signs of inflammation characterised by slight multifocal oedema in the submucosa, infiltration of some heterophils and lymphocytes in the lamina propria, and the presence of mucus and sloughed epithelial cells in the intestinal lumen (Figures 1E, F). These results indicate that LPS from *E. coli* O127:B8 induces sepsis and intestinal inflammation in rabbits, in contrast to *E. coli* O111:B4 (0.2 µg/kg, 90 min, IV), which induces an increase in the rectal temperature but does not induce intestinal inflammation in rabbits (Rebollar et al., 2002, 2003).

**Effects of LPS from *E. coli* O127:B8 on contractility of ileum**

We evaluated the effect of the LPS from *E. coli* O127:B8 on intestinal motor function at 2 levels: first, the direct effect on smooth muscle strips; and second, the effect on whole intestinal segments, to study the influence of the mucosa on motor function. LPS did not modify the amplitude of the spontaneous contractions in the muscle strips or whole intestinal segments (Figure 2A). However, LPS did increase the frequency of spontaneous contractions in whole segments of ileum, but not in muscle strips (Figure 2B), indicating that the actions of LPS on the mucosa might influence spontaneous intestinal contractions. LPS reduced the response to serotonin (Figure 2C) and increased the response to KCl (Figure 2E) in muscle strips, but not in whole segments, indicating a direct effect of LPS on the intestinal smooth muscle layer that regulates the serotonergic system and the K+ channels involved in neuromuscular transmission. Finally, LPS increased the response to acetylcholine only in whole segments (Figure 2D), indicating that the actions of LPS on the mucosa might influence the cholinergic transmission in the intestinal motor responses. In contrast, previous studies have demonstrated that LPS from *E. coli* serotype 0111:B4 (0.2 µg/kg, 90 min, IV) decreased the response to acetylcholine (Gonzalo et al., 2012) but increased the response to KCl (Rebollar et al., 2003; Gonzalo et al., 2010).

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Figure 1: (A and B) PCR amplification products and densitometric analysis of the mRNA expression of TLR4 in muscular and mucosal layers of ileum from rabbits treated with saline or lipopolysaccharide (LPS) from *E. coli* O127:B8 (100 µg/kg, 2 h, IV). Data are expressed as arbitrary units±mean standard error of 3 rabbits per group. Saline, LPS; **P<0.01 vs. saline. (C and D) Localisation of TLR4 in ileum from rabbits treated with LPS from *E. coli* O127:B8 by immunofluorescence. (E and F) Histological damage in the ileum of rabbits treated with (E) saline or (F) LPS from *E. coli* O127:B8. The inset of panel F shows an increase in inflammatory cell infiltrates in comparison with the inset of panel E. Representative sections from 3 different animals.
Figure 2: Effect of lipopolysaccharide (LPS) from *E. coli* O127:B8 (100 µg/kg, 2 h, IV) on the (A) amplitude and (B) frequency of spontaneous contractions, as well as the motor contractile responses induced by (C) serotonin (100 µM), (D) acetylcholine (100 µM), or (E) KCl (80 mM) in muscle strips or whole segments of rabbit ileum. Columns are mean values of amplitude (mN), frequency (contractions per min, cpm), or area under the curve (AUC, mN/s per g), and vertical bars indicate mean standard error from 4 rabbits per group. Saline, LPS *P<0.05; **P<0.01 vs. saline.
in whole segments of rabbit small intestine, indicating that LPS from *Escherichia coli* 0127:B8 alters the intestinal contractility of rabbits in a different way than LPS from *E. coli* 0111:B4.

LPS consists of 3 parts: (1) lipid A, a disaccharide acylated with fatty acid chains which is the toxic component of LPS; (2) the core region, a non-repetitive oligosaccharide (9 sugars in length) which can be subdivided into the inner and outer parts; (3) O-antigen, a serogroup-specific polysaccharide of repetitive oligosaccharide units (Huang et al., 2012).

The lipid A part is highly conserved in *E. coli*. It is responsible for inducing the immunopathogenic processes by binding the TLR4 that can lead to endotoxaemia-associated high mortality. The core of the *E. coli* strain is composed of the common hexoses, glucose (Glu), galactose (Gal), and N-acetyl glucosamine (GlcNAc) and the core regions are phosphorylated at the 2 inner heptoses. In *E. coli* LPS five distinct core structures have been characterised, termed K-12 and R1, R2, R3, R4. The serotype 0127:B8 possesses the R2 core type, whereas the serotype 0111:B4 strain possesses the R3 core type. The O-polysaccharide is linked to a sugar in the outer core. The O-antigen in *E. coli* LPS usually consists of 10-25 repeating units containing 2 to 7 sugar residues. The O-antigen polysaccharide chain length is highly variable among bacterial strains. In fact, the length of O-antigen of *E. coli* serotype O127:B8 is different from those of serotype 0111:B4 (Stenutz et al., 2006; Huang et al., 2012). The different compositions of the core and the O-antigen found in the *E. coli* LPS from serotypes 0127:B8 and 0111:B4 might explain the different effects of these serotypes on rabbit intestinal contractility.

**CONCLUSION**

In conclusion, our results show that LPS from *Escherichia coli* 0127:B8 induces a decrease in the mRNA expression of TLR4, an inflammatory response and changes in the contractility of rabbit ileum.

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