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Research Paper

Role of TLR4 and MAPK in the local effect of LPS on intestinal contractility

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Abstract

Objectives Lipopolysaccharide (LPS) has been shown to alter intestinal contractility. Toll-like receptor 4 (TLR4), K^+ channels and mitogen-activated protein kinases (MAPKs) have been proposed to be involved in the mechanism of action of LPS. The aim of this study was to determine the role of TLR4, K^+ channels and MAPKs (p38, JNK and MEK1/2) in the local effect of LPS on the acetylcholine (ACh)-induced contractions in rabbit small intestine *in vitro*.

Methods Segments of rabbit duodenum were suspended in the direction of longitudinal or circular smooth muscle fibres in a thermostatically controlled organ bath.

Key findings LPS (0.3 μg/ml) reduced the contractions induced by ACh (100 μM) in the longitudinal and circular smooth muscle of the duodenum after 90 min of incubation. Polymyxin (TLR4 inhibitor), SB203580 (p38 MAPK inhibitor), SP600125 (JNK1/2 inhibitor) and U0126 (MEK1/2 inhibitor) antagonized the effects of the LPS on ACh-induced contractions in duodenal smooth muscle. Incubation with the blockers of K^+ channels, TEA, apamin, charybdotoxin, iberiotoxin, glibenclamide or quinine, did not reverse the effect of LPS on ACh-induced contractions.

Conclusions These results suggest that the effect of LPS on ACh-induced contractions in the rabbit duodenum might be mediated by TLR4 and p38, JNK1/2 and MEK1/2 MAPKs.

Keywords gastrointestinal motility; K^+ channels; LPS; MAPK; TLR4

Introduction

Lipopolysaccharide (LPS) is an endotoxin present in the cell wall of Gram-negative bacteria. Many alterations associated with bacterial infections, such as fever, circulatory changes and damage to numerous organs, including the central nervous system, heart, kidneys, lungs, liver and gastrointestinal tract, are attributed to LPS.[1–4] LPS causes alterations in gastrointestinal motility both *in vivo* and *in vitro*.[5–9] Previous studies by our group[8] have reported an inhibitory effect of LPS on acetylcholine (ACh)-evoked contractions in intestinal segments. Alterations in gastrointestinal motility have been widely reported in response to the systemic administration of the endotoxin.[10,11] However, the mechanism by which local treatment with LPS alters intestinal motility needs further investigation.

The principal mechanism by which LPS is sensed is via an LPS-binding protein (LBP)–LPS complex and then signalling through the toll-like receptor 4 (TLR4)–MD-2 complex. However, other cell surface molecules also sense LPS; these include the macrophage scavenger receptor CD11b/CD18 and ion channels.[12]

Recognition and defence systems against bacterial infections are distributed throughout multicellular organisms. The mediation of cellular activation in response to LPS is known to occur through TLR4, a member of the toll receptor family.[13] When LPS binds to TLR4, multiple intracellular signalling pathways are activated, a process that is facilitated by two adapter proteins (MD-2 and CD14) and activated by the mitogen-activated protein kinases (MAPK).[12,14,15]

Protein kinases are key regulators of cell function that constitute one of the largest and most functionally diverse gene families. MAPKs are a family of Ser/Thr protein kinases widely conserved among eukaryotes and involved in many cellular programs such as cell proliferation, cell differentiation, cell movement and cell death.[16] The most extensively studied groups of vertebrate MAPKs to date are the extracellular signal-regulated kinase

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(ERK1/2), Jun-N-terminal kinase (JNKs) and p38 kinases. An abnormal activation of MAPK has been observed in pathological circumstances such as cancer,[17] inflammatory bowel disease[18,19] and sepsis.[10] Recently our group has proposed that the inhibition of the intestinal contractility induced by LPS is mediated by p38 and ERK MAPKs in rabbits treated with endotoxin.[10,20]

Most excitable cells express several types of K^+ channels. In fact, a number of different K^+ channels have been identified in the smooth muscle cells of the gastrointestinal tract.[21] We have previously described that Ca^{2+}-activated K^+ channels of small and high conductance, HERG K^+ channels and inward rectifier K^+ channels participate in the spontaneous contraction of rabbit small intestine.[22] However, it is unknown whether potassium channels, Toll-like 4 receptors or MAPKs participate in the local effects evoked by LPS *in vitro* on the intestinal contractility.

The aims of this study were to determine whether the local effects evoked by LPS *in vitro* on the ACh-induced contractions in rabbit small intestine are mediated: by (i) K^+ channels, (ii) Toll-like receptor 4 or (iii) MAPKs.

Materials and Methods

Drugs and solutions

The composition of the normal Krebs solution in mM was as follows: NaCl 120, KCl 4.7, $CaCl_2$ 2.4, $MgSO_4$ 1.2, $NaHCO_3$ 24.5, KH_2PO_4 1.0 and glucose 5.6, pH 7.4. ACh, LPS (from *Escherichia coli* serotype 0111 : B4), polymyxin B sulfate, tetraetylammonium chloride (TEA), apamin (AP), charybdotoxin (ChTX), iberiotoxin (IbTX), glibenclamide and quinine were purchased from Sigma (Madrid, Spain). 4-[5-(4-Fluorophenyl)-2-[4-(methylsulphonyl)phenyl]-1*H*-imidazol-4-yl]pyridine hydrochloride (SB-203580), anthra [1-9-cd]pyrazol-6(2*H*)-one (SP-600125) and 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene (U-0126) were acquired from Tocris (Madrid, Spain). SB-203580, SP-600125, U-0126 and glibenclamide were dissolved in dimethyl sulfoxide (DMSO). The solutions were diluted such that the final concentration of DMSO was <0.1% (v/v). This concentration of DMSO did not have effect on intestinal contractility. Apamin was dissolved in acetic acid. All of the other drugs were prepared in distilled water.

Animals

The handling, equipment used and sacrifice of animals complied with European Council legislation 86/609/EEC concerning experimental animal protection. All experimental protocols were approved by the Ethics Committee of the University of Zaragoza (Spain). Male New Zealand rabbits, 2–2.5 kg, were kept with standard rabbit fodder and free access to water.

Muscle contractility studies

After 24 h of fasting, the rabbits were humanely killed by a blow to the head. Pieces of rabbit duodenum were removed, washed, freed from mesenteric attachment and cut into smaller segments. Whole-thickness segments (10 mm long and 5 mm wide) were suspended in the direction of the longitudinal or circular smooth muscle fibres in a thermostatically controlled (37°C) organ bath (10 ml capacity) containing Krebs solution and continuously gassed with 95% O_2–5% CO_2. Each segment was connected to an isometric force transducer (Pioden UF1; Graham Bell House, Canterbury, UK) and stretched passively to an initial tension of 20 mN. Signal output of the mechanical activity was amplified (The Mac Lab Bridge Amp; AD Instruments Inc., Milford, MA, USA) with a range of 2 mV, recorded on a computer for later analysis using the Mac Lab System/8e computer program (AD Instruments Inc., Milford, MA, USA) and digitized at two samples per second per channel. Before testing, segments were allowed to equilibrate in Krebs solution for 45 min. During that time, the nutrient solution was changed every 20 min.

Each experimental protocol was systematically performed on eight segments of duodenum (4 longitudinal and 4 circular muscle) taken from the same rabbit and repeated in three or four different animals. Thus, each preparation served as its own control. Segments that did not show spontaneous activity were discarded.

After the equilibration period, we added ACh 100 μM to the bath, and the evoked contractile response was considered the control. To investigate the local effect of LPS on the longitudinal and circular smooth muscle of rabbit duodenum, the duodenum segments were then incubated for 90 min with Krebs or LPS (0.3 μg/ml) and, afterwards, ACh 100 μM was added to the bath. This second ACh response was compared with the control and expressed as a percentage. We examined the role of TLR4 and MAPKs in the LPS-induced effects by means of polymyxin (36 μM, a TLR4 inhibitor) and SB203580 (0.1 μM, a selective p38 inhibitor), SP600125 (0.1 μM, a selective JNK inhibitor) and U0126 (0.1 μM, a selective MEK1/2 inhibitor). These agents were added to the bath 15 min before incubation for 90 min with Krebs or LPS (0.3 μg/ml). The same protocol was performed with DMSO (SB203580, SP600125 and U0126 vehicle) to check that it had no effect *per se*.

To determine the participation of the different types of K^+ channels in the LPS-induced effects on ACh-induced contractions, we used different blockers of several K^+ channels: tetraetylammonium (5000 μM, a non-specific K^+ channel blocker), apamin (1 μM and 0.1 μM, a blocker of small-conductance Ca^{2+}-activated K^+ channels), charybdotoxin (0.01 μM, a selective blocker of intermediate- and large-conductance Ca^{2+}-activated K^+ channels), iberiotoxin (0.1 μM, a blocker of large-conductance Ca^{2+}-activated K^+ channels), glibenclamide (0.1 μM, a blocker of ATP-sensitive K^+ channels) and quinine (10 μM, a blocker of voltage-sensitive K^+ channels). These substances were added to the bath 15 min before the LPS (0.3 μg/ml) incubation for 90 min.

Data analysis and statistics

All of the intestinal segments included in the analyses showed spontaneous contractions. The ACh motor responses (MR) were measured as integrated mechanical activity (IMA) per second, expressed as mN/s and normalized per square millimetre of cross-sectional area (CSA, mm^2) as follows: MR = A1 − A0, where A is the integrated area per second per

mm^2 during either the first 3 min of response to ACh (A1) or the spontaneous motility, 3 min before adding ACh (A0).[8] The integrated area was calculated using a baseline of 0 mN. CSA was determined for each muscle strip using the equation CSA (mm^2) = mass (mg) [length (mm)·density (mg/mm^3)]$^{-1}$, where rabbit intestinal muscle density was assumed to be 1.05 mg/mm^3; the length and mass (wet weight) of each segment were measured upon completion of experiments.[23] The results were expressed as a percentage of the ACh control values (100%).

Results are expressed as mean ± SEM. Comparisons between means were made using one-way analysis of variance tests, and *P*-values were determined using the Scheffé *F*-test. $P < 0.05$ was considered statistically significant.

Results

Effect of lipopolysaccharide on acetylcholine-evoked contractions

ACh (100 μM) evoked contractions in the longitudinal and circular smooth muscle of the rabbit duodenum; contractions were not significantly modified after incubation with Krebs for 90 min (Figure 1a) or DMSO. The ACh-evoked contractions were inhibited in the presence of LPS (0.3 μg/ml, 90 min) compared with Krebs (90 min) in longitudinal and circular muscles (Figure 1b), as previously has been shown in our laboratory.

Effects of TLR4 inhibitor on acetylcholine-evoked contractions

Polymyxin (36 μM) added 15 min before endotoxin reversed the inhibitory effect of LPS on the ACh contractions in both longitudinal (Figures 1c and 2a) and circular muscle of rabbit duodenum (Figure 2a). Some duodenum segments were incubated with polymyxin, a TLR4 inhibitor, to check that it had no effect *per se* on ACh-evoked contractions. The incubation with polymyxin (36 μM) did not modify significantly the ACh-evoked contractions with respect to Krebs in both longitudinal (Figure 2a) and circular (Figure 2b) muscle of duodenum.

Role of K^+ channels in the inhibitory effect of lipopolysaccharide

The incubation with tetraetylammonium (5000 μM), apamin (1 μM), charybdotoxin (0.01 μM), iberiotoxin (0.1 μM), glibenclamide (0.1 μM) and quinine (10 μM) did not reverse the effect of LPS on ACh-induced contractions in both longitudinal and circular muscle of the rabbit duodenum (Table 1). Quinine does not produce *per se* effects on the ACh-induced contractions in both longitudinal and circular muscle of the duodenum (92 ± 24, $n = 8$; 102 ± 28, $n = 8$). Previously we have described that the other K^+ channels blockers do not cause *per se* effects either.[24]

Role of MAPKs in the inhibitory effect of lipopolysaccharide

SB203580 (0.1 μM), SP600125 (0.1 μM) and U0126 (0.1 μM) added 15 min before endotoxin reversed the inhibitory effect of LPS on the ACh contractions in both longitudinal and circular muscle (Figures 1 and 2) of rabbit duodenum. Some duodenum segments were incubated with the p38, JNK and MEK1/2 inhibitors to check that they had no effect *per se* on ACh-evoked contractions (protocol described in Materials and Methods). The incubation with SB203580, SP600125 and U0126 did not modify significantly the ACh-evoked contractions with respect to Krebs in both longitudinal (Figure 2a) and circular (Figure 2b) muscle of the duodenum.

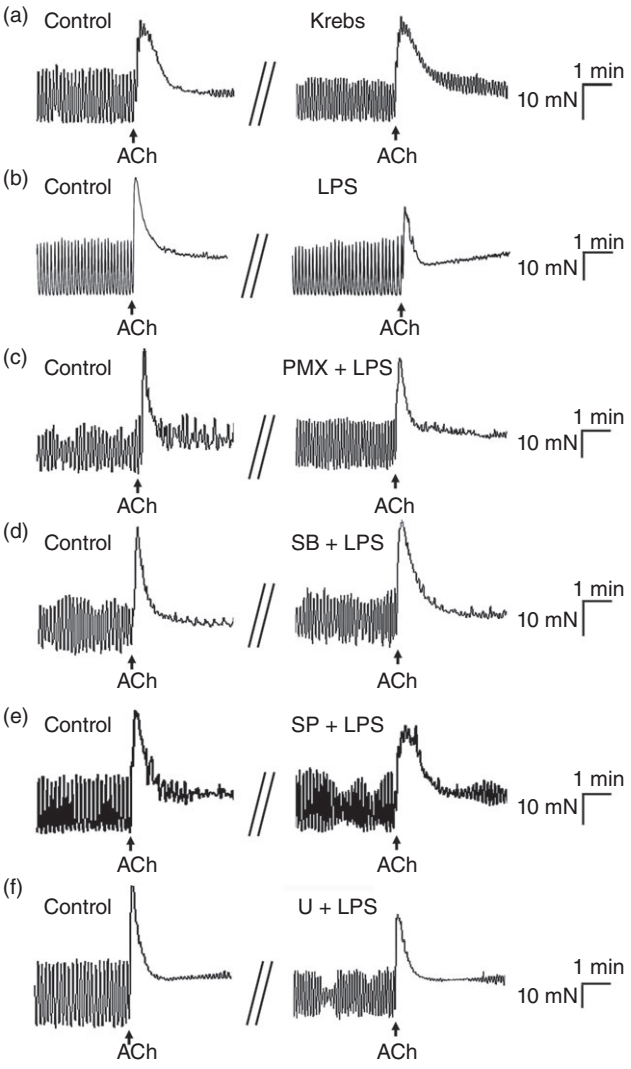

Figure 1 Effect of lipopolysaccharide (LPS) on duodenal contractility, and influence of inhibitors of TLR4 or MAPKs. Effect of the incubation for 90 min with Krebs (control) or LPS (0.3 μg/ml) on contractions evoked by acetylcholine (ACh, 100 μM) in longitudinal smooth muscle of rabbit duodenum. Influence of polymyxin B (PMX, 36 μM), SB203580 (SB, 0.1 μM), SP600125 (SP, 0.1 μM) or U0126 (U, 0.1 μM) added 15 min before the LPS (0.3 μg/ml). Arrowheads indicate addition of acetylcholine.

Discussion

Previous studies by our group[8] have reported a local inhibitory effect of LPS on the ACh-evoked contractions when

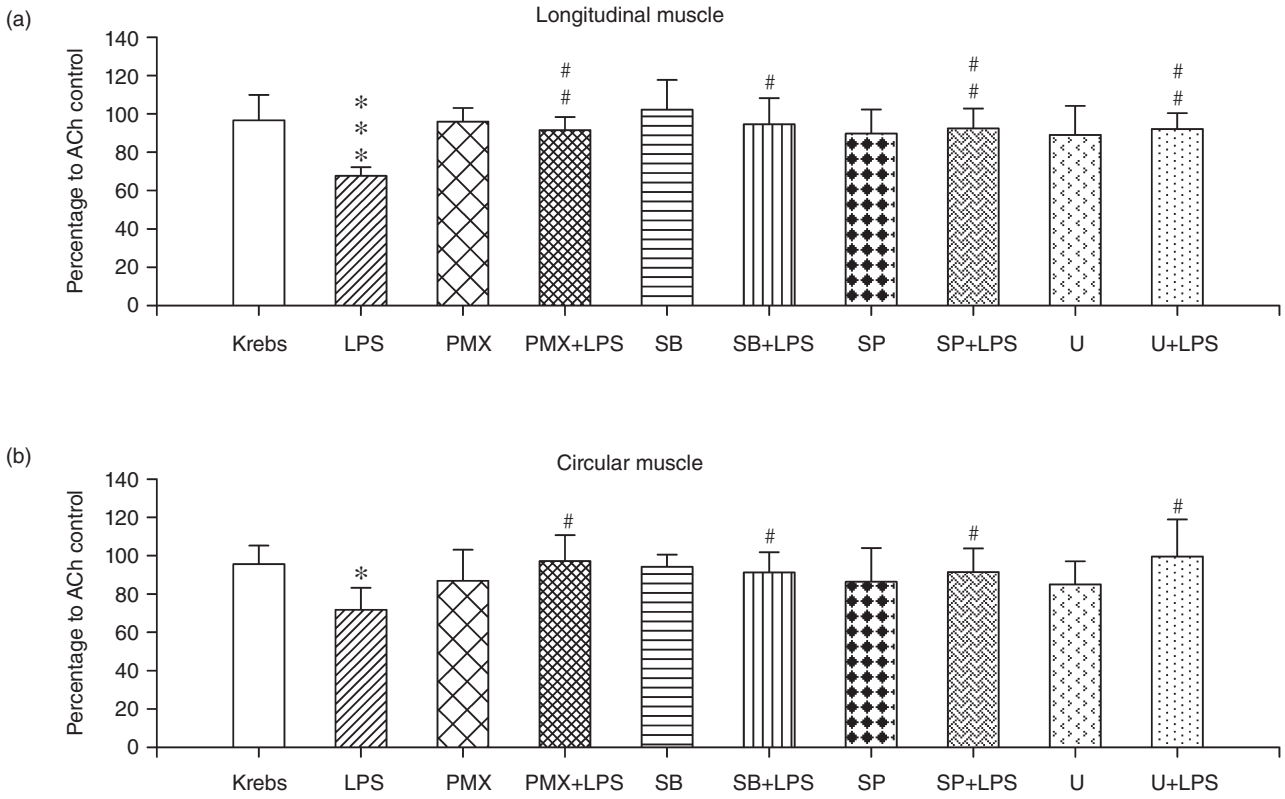

Figure 2 Effect of lipopolysaccharide (LPS) and inhibitors of TLR4 or MAPKs on duodenal contractility. Effect of the incubation for 90 min with Krebs (control) or LPS (0.3 μg/ml) on contractions evoked by acetylcholine (ACh, 100 μM) in longitudinal (a) and circular (b) smooth muscle of rabbit duodenum. Influence of polymyxin B (PMX, 36 μM), SB203580 (SB, 0.1 μM), SP600125 (SP, 0.1 μM) or U0126 (U, 0.1 μM) added 15 min before Krebs or LPS (0.3 μg/ml). Data are expressed as a percentage of the response to ACh control values (100%). Columns are mean values, and vertical bars indicate SEM. $^{*}P < 0.05$, $^{***}P < 0.001$ vs Krebs. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs LPS.

Table 1 Acetylcholine-induced contractions in longitudinal and circular smooth muscle of rabbit duodenum incubated for 90 min in Krebs solution or lipopolysaccharide

	Longitudinal muscle	Circular muscle
KREBS	96.8 ± 7.2 (11)	96.3 ± 7.7 (10)
LPS	61.0 ± 7.5 (9)*	64.6 ± 5.7 (12)*
TEA + LPS	70.0 ± 10.2 (8)*	65.6 ± 12.9 (8)*
AP + LPS	31.6 ± 10.9 (8)***	69.5 ± 8.4 (8)*
ChTX + LPS	51.6 ± 8.8 (12)***	61.3 ± 6.0 (11)***
IbTX + LPS	26.5 ± 6.5 (8)***	36.9 ± 7.3 (9)**
GB + LPS	52.1 ± 7.8 (9)***	60.0 ± 10.6 (9)*
Qn + LPS	72.1 ± 12.3 (12)**	76.0 ± 8.3 (10)**

Effect of TEA (5000 μM), apamin (AP, 1 μM), charybdotoxin (ChTX, 0.01 μM), iberiotoxin (IbTX, 0.1 μM), glibenclamide (GB, 0.1 μM) or quinine (Qn, 10 μM) added 15 min before lipopolysaccharide (LPS, 0.3 μg/ml). The values are the mean ± SE. Data are expressed as a percentage of response of acetylcholine (% of controls). The number of segments is in parentheses. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. Krebs.

rabbit intestinal segments were incubated with LPS *in vitro*. The main finding of the present study is that TLR4 and MAPKs but no K^+ channels are involved in the local effect of LPS on intestinal contractility.

LPS, as one of the most potent inducers of the immune system, is recognized by a complex cascade of extracellular 'pattern recognition receptors', which chaperone the LPS from the bacterial membrane to the transmembrane receptor TLR4.[25] We have studied the role of TLR4 in the local effect of LPS using the specific inhibitor polymyxin B sulfate. Our results show that TLR4 is involved in the mechanism of action of LPS on intestinal contractility. These results agree with another study in which TLR4 deletion significantly prevented intestinal muscle dysfunction in postoperative ileus.[26] TLR4-deficient mice are hyporesponsive to LPS[27] and smooth muscle, and the myenteric plexus cells of murine intestine have revealed expression of TLR4 in LPS-treated animals, showing a possible role of TLR4 in LPS-induced motility disturbances.[28] However, less information about the role of TLR4 in local treatment with LPS has been provided. The study of different receptor antagonists represents an useful tool for the treatment of gastrointestinal motility disorders.[29]

Various K^+ channels participate in intestinal spontaneous motility,[22] and different K^+ channels have been involved in LPS signalling.[30,31] The inhibition of LPS-induced cytokine production by the nonspecific K^+ channel blocker quinine has been described, showing a role for K^+ channels in LPS signal transduction.[32] LPS treatment changes the density of inwardly rectifying K^+ channels,[33] and the activation of

high-conductance Ca^{++}-activated K^+ channels by LPS in artery smooth muscle cells and in human alveolar macrophages[32,34] has been proposed. An abnormal activation of K^+ channels in vascular smooth muscle has been observed in animals with endotoxic shock and it has been suggested that an overproduction of nitric oxide causes the activation of large-conductance Ca^{++}-activated K^+ channels and ATP-sensitive K^+ channels that contribute to endotoxin-mediated vascular hyporeactivity.[35] Another study has shown that high-conductance Ca^{++}-dependent and voltage-dependent K^+ channels are involved in transmembrane signal transduction in macrophages as an early step and that the modulation of the channel by endotoxin is strongly sensitive to the conformation of lipid A. However apamin, a blocker of small-conductance Ca^{++}-activated K^+ channels, does not inhibit cytokine production.[36] Previously our group has described that K^+ channel blockers do not produce effects *per se* on the ACh-induced contractions[24] and in this study quinine does not cause any *per se* effect either. In the present work, we have observed that the incubation with different blockers of K^+ channels did not reverse the effect of LPS on ACh-induced contractions. These results suggest that the effects of LPS are not mediated by K^+ channels.

One pathway of intracellular activation induced by LPS is MAPKs.[12,25] In general, distinct stimuli activate mitogen-activated and stress-activated kinase subgroups with distinct cellular effects. Certain stimuli, such as LPS and TNF-α, activate multiple MAPKs in their target cells.[37,38] With the availability of specific kinase inhibitors, the importance of individual pathways to cellular responses can be determined. We used selective inhibitors of p38, JNK and MEK1/2 to determine which of these pathways contributed to LPS-induced intestinal disturbances. It has been reported that LPS activates all three MAPKs.[39] We have previously described that the inhibition of p38 MAPK improves intestinal disturbances induced in a rabbit endotoxaemia model[10] by intravenous LPS, but further investigation of the role of MAPK in the local effect of LPS is needed. In this study, the treatment with specific MAPK inhibitors reversed the effect of LPS on intestinal motility. This is in good agreement with other studies where MAPK inhibitors restored altered intestinal transit in burned rats[40] or the beneficial effect of antioxidants in the LPS-induced motility disorders associated with a reduction in MAPK activation.[11] In fact, antioxidants are being extensively used to restore the altered muscle response although sometimes they have effects *per se* on contractility.[41] MAPKs seem to be an important focus for the therapy of various diseases involving intestinal motility disorders such as inflammatory bowel disease[18] or postoperative ileus.[42]

We have previously studied the expression of some proinflammatory enzymes such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in segments of duodenum incubated with LPS, and we found that their expression was not modified by LPS.[43] Nevertheless, COX-2 expression was modified in the duodenum of rabbits intravenously treated with LPS.[44] According to these data, the LPS-effects and the pathways involved change depending on whether the administration of LPS is local or systemic. It has been suggested that the K^+ channels are activated by an LPS-induced overproduction of nitric oxide (i.e. as a secondary or even tertiary step of LPS signalling) and that they contribute to the endotoxin-mediated damage.[35] We have observed that iNOS and, consequently, nitric oxide production was not increased in our model of local treatment with LPS[43] and therefore these data agree with our results such that the K^+ channels are not involved in the local effect of LPS.

Conclusions

In conclusion, our results show that the effect of LPS administered locally on ACh-induced contractions in the rabbit duodenum might be mediated by TLR4 and p38, JNK1/2 and MEK1/2 MAPKs. This study showed no evidence of a role of K^+ channels in the effect of LPS in the intestinal contractility *in vitro*.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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