Genetics and Molecular Research

Intestinal alkaline phosphatase and sodium butyrate may be beneficial in attenuating LPS-induced intestinal inflammation

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Genet. Mol. Res. 15 (4): gmr15048875
Received June 10, 2016
Accepted July 20, 2016
Published October 17, 2016
DOI http://dx.doi.org/10.4238/gmr15048875

ABSTRACT. In this study, we evaluated the effect of intestinal alkaline phosphatase (IAP) and sodium butyrate (NaBu) on lipopolysaccharide (LPS)-induced intestinal inflammation. Intestinal alkaline phosphatase and RelA/p65 (NF-κB) gene expressions in porcine jejunum explants were evaluated following exposure to sodium butyrate (NaBu) and essential oil from Brazilian red pepper (EO), alone or in combination with NaBu, as well as exogenous IAP with or without LPS challenge. Five piglets weighing approximately 20 kg each were sacrificed, and their jejunum were extracted. The tissues were segmented into 10 parts, which were exposed to 10 treatments. Gene expressions of IAP and RelA/p65 (NF-κB) in jejunal explants were evaluated via RT-PCR.

We found that EO, NaBu, and exogenous IAP were able to up-regulate endogenous IAP and enhance RelA/p65 (NF-κB) gene expression. However, only NaBu and exogenous IAP down-regulated LPS-induced inflammatory response via RelA/p65 (NF-κB). In conclusion, we demonstrated that exogenous IAP and NaBu may be beneficial in attenuating LPS-induced intestinal inflammation.

Key words: Brazilian red pepper essential oil; *Ex vivo*; Swine; Intestinal alkaline phosphatase; Intestinal explants; Sodium butyrate;

INTRODUCTION

Bacterial lipopolysaccharides (LPS), the major components of the outer membranes of Gram-negative bacteria, are potent inflammatory inducers (Islam and Pestka, 2006; Bortoluzzi et al., 2016), and may be associated with inflammatory bowel disease (IBD) and sepsis. The resident gut microbiome is one of the most abundant sources of LPS in humans and animals. Dysbiosis of gut microbiota has been linked to intestinal inflammation (Malo et al., 2014). There are much difficulties associated with studying gene expression in human tissues, especially in the intestinal tract. Owing to the similarities in intestinal physiology between pigs and humans, studies that examine the relationship between immune inducers and nutritional influences are preferably carried out in pig models (Roura et al., 2016).

The viability of *ex vivo* techniques has been recently investigated to evaluate the modulatory effects of natural agents on immune responses in intestinal explants (Bahar et al., 2012; Leonard et al., 2012). Furthermore, *ex vivo* techniques applied in these studies may offer a quick, less expensive, specific, and reliable experimental model to determine the effects of immune inducers or feed contaminants on intestinal mucosa (Basso et al., 2013; Bortoluzzi et al., 2016).

Herbal extracts and organic acids are natural compounds that have been associated with enhanced gut health, as they can act as modulators of the microbiota and stimulators of intestinal function (Lange et al., 2010). The phytochemical properties of these natural anti-inflammatory substances suggest that they can be used as potential therapeutic agents to attenuate unwanted immune responses (Fei et al., 2014). For example, essential oil extracted from Brazilian red pepper (EO) (*Schinus terebinthifolius* Raddi) has been shown to promote gut health (Gois et al., 2016) by acting as an antioxidant (Bendaoud et al., 2010), antimicrobial (de Lima et al., 2006), anti-inflammatory (Barbosa et al., 2007), and anti-ulcerogenic agent (Carlini et al., 2010, Carvalho et al., 2013). However, no studies have evaluated its effects on intestinal responses to inflammatory agents such as bacterial LPS.

The short-chain fatty acid sodium butyrate (NaBu), derived from butyric acid, plays regulatory roles in intestinal function (Canani et al., 2011), including reduction of inflammatory responses through elimination of NF-κB constitutive p50 dimer in HT-29 cells (Inan et al., 2000). Recently, *in vitro* studies confirmed that NaBu up-regulates the gene expression and activity of the brush border enzyme intestinal alkaline phosphatase (IAP) (Malo et al., 2006; Bol-Schoenmakers et al., 2010). IAP is an isoenzyme that hydrolyzes phosphate monoesters at alkaline pH (Sussman et al., 1989), and is able to detoxify bacterial components such as LPS (Koyama et al., 2002; Bates et al., 2007), flagellin, and CpG DNA (Chen et al., 2010) via dephosphorylation. Furthermore, it can prevent and reduce intestinal inflammation and bacterial translocation (Martínez-Moya et al., 2012).

We hypothesized that NaBu and/or EO could up-regulate IAP, enhance gene expression of NF-κB dimer RelA/p65, and hence attenuate LPS-induced inflammatory responses in *ex vivo* porcine jejunum tissues. The aim of this study was to evaluate the gene expression of IAP and RelA/p65 (NF-κB) exposed to NaBu and/or EO, with or without LPS challenge, using an *ex vivo* model of intestinal explant.

MATERIAL AND METHODS

All animal handling procedures were approved by the Purdue Animal Care and Use Committee. Jejunal tissues (30 cm in length) were collected from the stomach of five pigs weighing approximately 20 kg. The jejunum was segmented into 10 parts (2 cm^2) from each pig, and was randomly assigned to one of the treatments. Feed additives were supplied according to the following 10 treatments: T1 - control (without additives); T2 - 5 mM NaBu (≥98,5%; Sigma Aldrich, St. Louis, MO, EUA); T3 - 2 ppm EO from Brazilian red pepper fruit (*S. terebinthifolius* Raddi; Agro Rosa Ltd Company, São Mateus, ES, Brazil); T4 - 5 mM NaBu + 2 ppm EO; T5 - 4 U/mL Calf IAP (Sigma Aldrich); T6 - 10 mg/mL *Escherichia coli* LPS; T7 - 10 mg/mL *E. coli* LPS + 5 mM NaBu; T8 - 10 mg/mL *E. coli* LPS + 2 ppm EO; T9 - 10 mg/mL *E. coli* LPS + 5 mM NaBu + 2 ppm EO; T10 - 10 mg/mL *E. coli* LPS + 4 U/mL IAP. The major components found in EO fruit were described by Gois et al. (2016), and all experimental procedures were performed according to the protocol outlined by Bortoluzzi et al. (2016).

Gene expression analyses were performed via real-time PCR; levels of IAP (Lackeyram et al., 2010) and RelA/p65 (de los Santos et al., 2007) were evaluated with GAPDH as the reference gene (Smith et al., 2011). Relative mRNA expression was calculated according to the methods proposed by Livak and Schmittgen (2001). All experiments were replicated in two plates, and samples were analyzed in duplicates (four CT values for each treatment).

For statistical analysis, each jejunal explant collected from the piglets (10 explants/piglet) was considered as one experimental unit (N = 5 replicates/treatment). The effect of treatments on gene expression levels was assessed using the Student *t*-test with unequal variance. Significance was determined to be $P \leq 0.05$.

RESULTS

LPS induced gene expression of IAP and RelA/p65 (NF-κB)

The ability of LPS to induce inflammatory response is essential to evaluate the effectiveness of the *ex vivo* model used in this experiment, and to investigate the effect of the proposed treatments. Our model showed that the porcine jejunum tissue was responsive to LPS challenge, resulting in up-regulation ($P < 0.05$) of IAP (6.9 ± 0.73-fold higher than control) (Figure 1). Furthermore, RelA/p65 showed enhanced gene expression upon LPS treatment (NF-κB) (4.35 ± 0.3-fold higher than control) (Figure 2).

NaBu, EO, NaBu+EO, and exogenous IAP up-regulated IAP gene expression

Regulation of IAP by various treatments in intestinal explants is shown in Figure 1. NaBu treatment resulted in an increase ($P < 0.05$) in the relative mRNA expression of IAP by 8.21 ± 1.15-fold as compared with control treatment. In addition, EO (7.07 ± 0.98-fold higher

than control) and combination treatment of NaBu+EO (8.7 ± 0.76 fold-higher than control) were also able to up-regulate ($P < 0.05$) IAP gene expression. Furthermore, exogenous IAP further increased IAP gene expression ($P < 0.05$; 7.41 ± 1.44-fold), suggesting that IAP in the intestinal lumen acts as stimulator agent to cellular IAP expression.

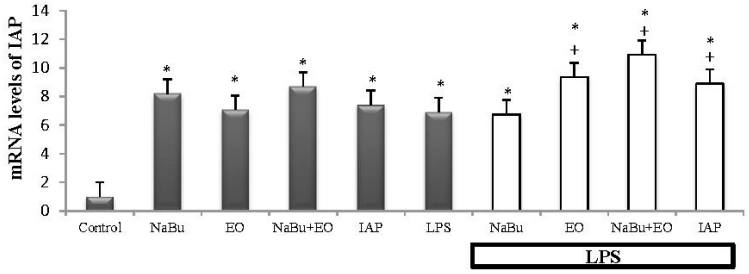

Figure 1. Gene expression of intestinal alkaline phosphatase (IAP) in porcine jejunum induced by different treatments as determined by qRT-PCR (expressed as fold higher than control based on $2^{-\Delta\Delta Ct}$, using GAPDH as reference gene). *$P < 0.05$ control *vs* treatments. $^{+}P < 0.05$ LPS *vs* additive + LPS.

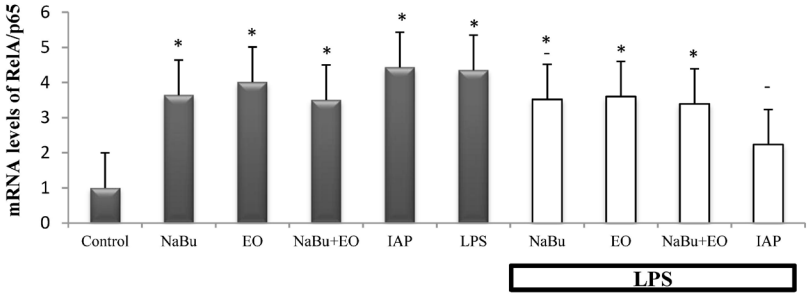

Figure 2. Gene expression of RelA/p65 (NF-κB) in porcine jejunum induced by different treatments as determined by qRT-PCR (expressed as fold higher than control based on $2^{-\Delta\Delta Ct}$, using GAPDH as reference gene). *$P < 0.05$ control *vs* treatments. $^{-}P < 0.05$ LPS *vs* additive + LPS.

Treatments complementarily up-modulated IAP gene expression in LPS presence

The relative mRNA levels of IAP from porcine explants treated with LPS+EO, LPS+NaBu+EO, and LPS+IAP were significantly higher ($P < 0.05$) as compared with that of LPS-only treatment (36, 58, and 29%, respectively). This suggests that these combination treatment have synergistic effects (Figure 1). However, there was no difference ($P > 0.05$) in gene expression of IAP when tissues were exposed to LPS+NaBu, as compared to that observed with LPS treatment.

RelA/p65 (NF-κB) gene expression was up-regulated by the treatments

The *ex vivo* findings in this study showed higher ($P < 0.05$) relative mRNA levels of RelA/p65 (NF-κB) in porcine explants exposed to NaBu (3.64 ± 0.52-fold higher than control), EO (4.01 ± 0.4-fold higher than control), NaBu+EO (3.50 ± 0.68-fold higher than control), and exogenous IAP (4.43 ± 0.5-fold) as compared with that of the control (Figure 2).

NaBu and exogenous IAP suppressed gene expression of RelA/p65 (NF-κB) in response to LPS challenge

The anti-inflammatory effect of the treatments was investigated in porcine jejunum tissue during LPS challenge (Figure 2). When tissues were exposed to LPS+NaBu, LPS+EO, and LPS+NaBu+EO, the expression of RelA/p65 (NF-κB) was up-regulated and was 3.52 ± 0.46-, 3.6 ± 0.68-, and 3.39 ± 0.97-fold higher than that of the control, respectively. However, LPS+IAP did not lead to up-regulation of RelA/p65(NF-κB). When compared to LPS treatment, explants exposed to LPS+NaBu and LPS+IAP showed suppressed LPS stimulation ($P < 0.05$), as demonstrated by reduced mRNA levels of RelA/p65 (NF-κB). Lastly, only exogenous IAP was capable of down-regulating LPS-induced inflammatory responses, ($P > 0.05$), as assessed by relative mRNA expression of RelA/p65 (NF-κB) as compared with that of the control.

DISCUSSION

The gastrointestinal tract is continuously exposed to various microorganisms. The Toll-like receptor 4 (TLR4) recognizes bacterial LPS as pathogen-associated molecular patterns (PAMPs) (Takeda and Akira, 2004), and stimulates the immune response via activation of NF-κB (Bol-Schoenmakers et al., 2010). The proteins p50 and p65 are NF-κB subunits that are highly expressed during signaling cascades (Baeuerle and Henkel, 1994; Goldberg et al., 2008). While there are several mechanisms by which inflammatory processes may occur, the common final response to NF-κB signaling is the release of cytokines, chemokines, and the recruitment of inflammatory cells (Berkes et al., 2003).

Recently, the role of IAP in detoxifying LPS and reducing the inflammatory response has been investigated (Koyama et al., 2002; Bates et al., 2007; Bol-Schoenmakers et al., 2010; Chen et al., 2011). It has been demonstrated that early weaning of piglets reduces the expression of IAP mRNA, and may predispose animals to enteric infections and diarrhea events, resulting in poor health and impaired growth (Lackeyram et al., 2010). In mice, the activity of alkaline phosphatase increases following colitis induction, which mimics IBD conditions in humans; exogenous IAP treatment reduced inflammatory markers and bacterial translocation (Martínez-Moya et al., 2012). However, there are no reports regarding the ability of exogenous IAP to regulate endogenous IAP expression. A few studies have suggested that herbal extracts and sodium butyrate may play a role in regulating IAP expression (Malo et al., 2006; Levkut et al., 2011). The present investigation demonstrated that EO, NaBu and exogenous IAP all up-regulate intestinal levels of IAP, which can contribute to the maintenance of local intestinal immunity.

The modulatory effect of NaBu on the immune response was confirmed in the present study when jejunal explants exposed to NaBu showed enhanced gene expression of RelA/p65 NF-κB. Activation of NF-κB is dependent on its translocation to the nucleus. In a previous study, NaBu reduced p50 NF-κB in the nucleus without affecting p50 level in the cytoplasm (Inan et al., 2000). LPS, IL-1β, and TNF-α can induce translocation of cytoplasmatic NF-κB into the nucleus, resulting in its activation. Bol-Schoenmakers et al. (2010) reported that following LPS challenge, cells exposed to the IAP up-regulator, NaBu, showed suppressed NF-κB. However, no further investigation was carried out on the NF-κB dimer produced in these LPS-induced cells. Other studies demonstrated that IL-1β treatment enhanced p65 expression,

while suppression of p65 was observed in TNF-α-treated cells exposed to NaBu (Inan et al., 2000). In the present study, we also showed reduced NF-κB activity in LPS presence. However, while NaBu blunted LPS and TNF-α induction, NF-κB activity was not reduced to basal levels, and its effect upon IL-1β seemed to be limited. Although there is a difference between p50- and p65-pathways, a possible explanation is that a part of the p50-p65 complex remains bonded after NaBu treatment, which can still activate gene transcription (Inan et al., 2000).

Pre-treatment of epithelial cell lines with NaBu before exposure to a PAMP that mimics bacterial LPS resulted in enhanced IL-8 production, which may be mediated by NF-κB inactivation. NaBu silenced IL-8 induction following LPS challenge via overexpression of A20, a protein that down-regulates NF-κB signaling (Weng et al., 2007). In the present study, the reduction of p65 and increased IAP expression in response to LPS+NaBu treatment suggest that, at least in part, the overexpression of IAP may down-regulate NF-κB signaling. The mechanism of action was reported be through histone hyperacetylation (Malo et al., 2006). Other studies examining the effects of NaBu on intestinal inflammation with regard to other pathways were summarized by Canani et al. (2011). Taken together, NF-κB activation can be differentially modulated depending on various inductors (e.g., IL-1β, TNF-α, LPS), and its anti-inflammatory effects could be mediated through up-regulation of IAP and/or A20, both of which down-regulate NF-κB signaling. Indeed, the modulation of epithelial response induced by NaBu can attenuate inflammatory events according to severity of cell damage (Bol-Schoenmakers et al. 2010).

Herbal extracts (e.g., seaweed extract, borneol oil, ciwujia dried roots) have been reported to be enhancers (Leonard et al., 2012) and attenuators (Juhás et al., 2008; Bahar et al., 2012; Fei et al., 2014) of immune responses. Further, essential oils (e.g., oregano and thymol) (Jang et al., 2007; Levkut et al., 2011) and dietary spices (black pepper, piperine, red pepper, capsaicin, and ginger) (Prakash and Srinivasan, 2010) have been shown to modulate IAP activity. The nonpolar property of these spices can facilitate interaction with lipids and hydrophobic portions of proteins, which was suggested to increase the intestinal brush border membrane fluidity, digestive enzymes, protein transporters, and nutrient absorption (Prakash and Srinivasan, 2010). In our study, we found that EO was able to up-regulate jejunal IAP mRNA. Nevertheless, this regulation was not able to suppress LPS-induced inflammatory response. It is possible that greater dose of EO is needed to silence LPS-induced intestinal inflammation via IAP modulation.

To the best of our knowledge, this is the first study reporting the combined effect of NaBu and EO in enhancing intestinal immune response and IAP production. We initially hypothesized that this combination therapy could positively complement the anti-inflammatory effects of NaBu. However, when tissue samples were exposed to LPS, production of RelA/p65 (NF-κB) in response to NaBu+EO was comparable to that of the control.

The *ex vivo* model used in this investigation has been well accepted as a valid method both to test injury induction from potential mitigating agents (Bahar et al., 2012; da Silva et al., 2014; Bortoluzzi et al., 2016). Indeed, we saw that intestinal explants could respond directly to exogenous inductors such as LPS, natural substances, and brush border enzyme. Under *in vitro* conditions, since diets between animals and humans differ, the nutrients consumed may also differentially modulate intestinal mucosa responses. Furthermore, diets alter the intestinal microbiota, which also can promote a variety of mucosal responses. To reduce this limitation, *ex vivo* studies can evaluate acute responses of intestinal explants from animal that received different diets. This allows for better understanding of the

interactions between food, microbiota, natural toxins, preventive and therapeutic agents, and host responses. To summarize, knowledge from *ex vivo, in vivo*, and genetic studies could all contribute to maintenance of intestinal health.

In this study, we found evidence to suggest that dietary supplementation with exogenous IAP or natural agents is able to maintain the expression and activity of IAP in the intestinal mucosa, and enhances intestinal immunity responses against bacterial-induced inflammation. Reducing LPS toxicity is an emerging strategy to reduce intestinal inflammation. Exogenous IAP is a beneficial agent that acts to detoxify LPS, thus improving intestinal health.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEGMENTS

Antonio Diego Brandão Melo thanks the Science Without Borders Program (CNPq) from the Brazilian government for financing his internship at Purdue University. The authors also would like to thank the Purdue University and Pontifícia Universidade Católica do Paraná (PUCPR) for funding this study.

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