Enterococcus faecium HDRsEf1 Represses CYP3A29 Expression in the Intestine through the TLR1/2-induced A20 to attenuate the NF-κB/RXR-α Signaling

Yucheng He
Huazhong Agriculture University
Yue Li
Huazhong Agriculture University
Yinghui Gong
Huazhong Agriculture University
Ying Wang
Huazhong Agriculture University
Xiue Jin
Huazhong Agriculture University
Deshi Shi
Huazhong Agriculture University
Xiliang Wang (wxl070@mail.hzau.edu.cn)
Huazhong Agriculture University https://orcid.org/0000-0001-7892-8018

Research

Keywords: probiotic, cytochrome P450, CYP3A29, RXR-α, NF-κB, TLR

Posted Date: March 5th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-16231/v1

Abstract

Background CYP3A29 is crucial for drug metabolism in pigs and an important research model of human CYP3A4. Recent studies have shown that some probiotics can regulate the expression of CYP3A in mammals, but the mechanisms underlying their action are unclear. This study explored whether Enterococcus faecium HDRsEf1 could modulate the expression of CYP3A29 in pig intestine and potential mechanisms.

Results we found that HDRsEf1 significantly reduced CYP3A29 expression in pig intestinal tissues and epithelial cells, dependent on cell-cell contact. In IPEC-J2 cells, HDRsEf1 treatment decreased the transcription activity of the CYP3A29 promoter, RXR-α expression and abrogated or mitigated the RXR-α or PXR over-expression-increased CYP3A29 expression. Both RXR-α/PXR over-expression synergistically increased CYP3A29 expression while RXR-α or PXR silencing reduced CYP3A29 expression. Co-immunoprecipitation revealed that RXR-α directly interacted with PXR. HDRsEf1, like a NF-kB inhibitor, significantly decreased the NF-kBp65 activation, RXR-α and CYP3A29 expression, which were abrogated by RXR-α silencing. The down-regulated CYP3A29 expression by HDRsEf1 was associated with increased A20 expression, which were abrogated by TLR1/2 silencing.

Conclusions HDRsEf1 inhibits the expression of CYP3A29 through the TLR1/2-induced A20 to attenuate the NF-κB/RXR-α signaling in pig intestinal tissues. Our findings may provide new insights into the mechanisms by which probiotics regulate the expression of CYP450s, and suggest potential risks in the clinical application of probiotics

Introduction

Drug metabolism in the intestine or intestinal first-pass metabolism can cause low bioavailability of some oral drugs, which depends on the contents of relevant enzymes in the gastrointestinal lumen and epithelium as well as bacterial enzymes (van Herwaarden et al., 2009; van Waterschoot et al., 2009). Probiotics can modulate gut microbiota and regulate immune responses, benefiting patients with intestinal diseases, diabetes, tumors and obesity(Marchesi et al., 2016). Probiotics have been widely used in humans and animals,and probiotics may alter the expression of intestinal drug-metabolizing enzymes (DMEs) to change the bioavailability of some oral drugs. However, the effect of probiotics on the expression of intestinal DMEs and their mechanisms have not been clarified. Accordingly, it is important to understand the effect and mechanisms of probiotics on the expression of intestinal DMEs.

Mammalian cytochrome P450s (CYP450s) are crucial for the oxidative metabolism of xenobiotics, including therapeutic drugs, environmental carcinogens and toxins(Guengerich, 1997). CYP3A4, one of the DMEs in humans, can metabolize > 50% of clinical drugs (Rodriguez-Antona et al., 2010). Pigs have a high similarity in physiology and anatomy to humans and become an important animal model for evaluating new drugs (Shang et al., 2013; Skaanild, 2006). Porcine CYP3A29 accounts for 30% of total CYP proteins and the major CYP3A activity in pig liver microsomes (Wu et al., 2016). Pig CYP3A29 and

human CYP3A4 have similar tissue distribution, pharmacokinetic characteristics and regulatory mechanisms, and pig CYP3A29 is a suitable model for research of human CYP3A4 (Anzenbacher et al., 1998; He et al., 2018).

The nuclear receptors (NRs), such as retinoid X receptor alpha (RXR-α), pregnane X receptor (PXR), vitamin D receptor (VDR) and the constitutive androstane receptor (CAR), are important for regulating the expression of CYP450s(Zordoky and El-Kadi, 2009). The RXR-α can directly interact with PXR, VDR or CAR to form heterodimers and bind to the CYP450s gene promoter to regulate their expression(Lefebvre et al., 2010). For example, the VDR/RXR-α heterodimer can bind to the CLEM4-ER6 motif to regulate the expression of CYP3A4(Pavek et al., 2010), and the CAR/RXRα and PXR/RXR-α heterodimers can interact with pER6 and dXREM to regulate the expression of CYP3A4, respectively (Chen et al., 2010; Toriyabe et al., 2009). Our previous study has shown that the PXR/RXR-α can bind to the CYP3A29 promoter to induce its expression in porcine liver HepLi cells (Zhou et al., 2016). Apparently, RXR-α expression is critical for the expression of CYP3A in the digestive system.

The nuclear factor-kappa B (NF-κB) signaling is crucial for inflammation, immunity, cell proliferation and apoptosis(Pereira and Oakley, 2008). It can regulate the expression of CYP450s by directly binding to the CYP450 promoters,or indirectly regulate the expression of NRs (Lee et al., 2000; Zhou et al., 2016; Zordoky and El-Kadi, 2009). In addition, the NF-κB can also enhance the activity of CYP450 at the post-transcriptional level by stabilizing CYP450 proteins (Zangar et al., 2008). Interestingly, previous studies have revealed that some probiotics can inhibit the NF-κB activity (Kawano et al., 2019; Sun et al., 2017). However, whether and how probiotics could modulate the expression of CYP450s, such as CYP3A29 in pig intestinal tissues, have not been clarified.

Toll-like receptors (TLRs) act as a type of pattern recognition receptors (PRR) in the innate immune system and TLRs can recognize microbial components to initiate immune responses. TLR2 can directly interact with TLR1 or TLR6 to form a heterodimer and recognize conserved molecular patterns (such as peptidoglycan, lipoteichoic acid, etc.) on the cell wall of Gram-positive bacteria(Jin et al., 2007; Takeuchi et al., 1999). Engagement of TLR2 by the cell wall components of some probiotics can up-regulate the expression of some NF-κB inhibitors, such as A20 (tumor necrosis factor-α-induced protein 3), IRAK-M and Tollip, to inhibit inflammation(Kawano et al., 2019; Sun et al., 2017). Actually, A20 is a deubiquitinating enzyme and can inhibit the NF-kB activation (Boone et al., 2004). Our previous study indicates that Enterococcus faecium HDRsEf1 enhances the expression of occluding, the intestinal tight junction protein, by activating the TLR2 (Yan et al., 2018). Accordingly, we hypothesize that HDRsEf1 can modulate the expression of CYP3A29 in pig intestinal tissues by modulating the NB-kB signaling.

In this study, we explored the effect of HDRsEf1 on CYP3A29 expression and potential mechanisms in pig intestinal tissues and epithelial IPEC-J2 cells. We found that HDRsEf1 decreased the expression of CYP3A29 in pig intestinal tissues by activating the TLR2 to up-regulate A20 expression, inhibiting the NF-kB activation to attenuate RXR-α expression.

Materials And Methods

Special chemicals, and reagents

The special reagents included Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin and Lipofectamine 2000 transfection reagent (Gibco, Life Technologies, Grand Island, NY, USA); 5,6-Dichlororibosidyl-benzimidazole (DRB) and BAY11-7082 (Sigma-Aldrich, St. Louis, MO, USA); monoclonal antibodies against CYP3A29, PXR, RXR-α, CAR and VDR (Santa Cruz Biotechnology, Santa Cruz, CA, USA), NF-κBp65, Phospho-NF-κB p65 (Cell Signaling Technology, Beverly, MA, USA), β-actin and HRP-conjugated secondary antibodies (ABclonal Technology, Wuhan, China); and the dual-luciferase reporter assay system (Promega, Madison, WI, USA).

Bacterial preparations, cell culture and treatment

Enterococcus faecium HDRsEf1 is novel probiotic strain (CCTCC NO: M2011031) (Yan et al., 2018). The HDRsEf1 bacteria were grown in De Man Rogosa Sharpe (MRS) media (Difco, Sparks, USA) at 37 °C for 18 h,and collected by centrifugation. After being washed twice with phosphate buffered saline (PBS), the bacteria were re-suspended in antibiotic-free DMEM at 1 × 108 colony forming units (CFU) ml-1.

Porcine small intestinal epithelial IPEC-J2 cells were obtained from BeNa Culture Collection (Beijing, China), and cultured in 10% FBS DMEM. To determine the effect of HDRsEf1, IPEC-J2 cells (2 × 105 cells/well) were challenged in triplicate with vehicle or 106, 107, or 108 CFU/mL HDRsEf1 for 2, 4 or 6 h, respectively. Furthermore, IPEC-J2 cells (2 × 105 cells) were cultured in the bottom chambers of 12-well transwell plates (0.2-μm-pore-size, Corning, NY, USA) and HDRsEf1 cells (108 CFU/well)were cultured in the upper chamber to determine the cell-cell contact independence. Their cell viability was quantified by Cell Counting Kit-8 assay (Biosharp, Hefei, China) and CYP3A29 expression were examined by qRT-PCR and Western blot. In addition, IPEC-J2 cells were treated with 108 CFU/mL HDRsEf1 in the presence or absence of 5 μM DRB and/or 10 μM BAY11-7082 and the relative levels of CYP3A29 mRNA transcripts were quantified.

Animals and procedures

Female (Landrace × Large White) pigs at an age of 30 days with similar body weights were obtained from COFCO and randomly fed with the control corn and soybean meal-based diet (Hu et al., 2019) or the control diet containing HDRsEf1 (106 CFU/g) through a feeder for 30 days. Some pigs (4 each group) were randomly selected, anesthetized and sacrificed by jugular puncture (Hu et al., 2017). Their segmented jejunal tissues at the approximately middle intestine were collected for subsequent experiments. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Huazhong Agricultural University.

Quantitative Real-Time PCR (qRT-PCR)

The relative levels of target gene to the control β-actin mRNA transcripts were determined by qRT-PCR using specific primers (Table S1) and iQ™ SYBR Green PCR Supermix in the Bio-Rad CXF real-time PCR detection system (Bio-Rad, CA, USA) after we extracted total RNA from individual groups of cells with the TRIzol reagent (Invitrogen) and reversely transcribed (RT) into cDNA using the Superscript reverse transcriptase (Takara, Otsu, Japan). The data were analyzed by 2-ΔΔCt method.

Western blotting

We extracted the jejunal tissue proteins as our previously described (Yan et al., 2018) and the proteins from individual groups of cells after lyzing IPEC-J2 cells with RIPA Buffer (Pierce, Rockford, IL, USA). Similarly, we also extracted nuclear and cytoplasmic proteins from individual groups of cells using specific extraction kit (EpiZyme, Shanghai, China). The protein samples (20 μg/lane) were separated by SDS-polyacrylamide gel electrophoresis on 10% gels and transferred onto polyvinylidene fluoride membranes. After being blocked with 5% non-fat dry milk in TBST, the membrane was incubated with primary antibodies at 4°C overnight and reacted with secondary antibodies, followed by visualizing using ECL chemiluminescence system (Bio-Rad).

Transfection and luciferase assay

IPEC-J2 cells were maintained in Opti-MEM medium until 80% of confluence and transfected with pGL3-CYP3A29 that contained the CYP3A29 promoter or the NF-kBp65 promoter using Lipofectamine 2000 for 18 h. Furthermore, IPEC-J2 cells were co-transfected with pGL3-3A29-2007 and plasmid for Renilla luciferase expression for 18 h. The cells were treated with, or without, 108 CFU/mL HDRsEf1 for 6 h and the CYP3A29 promoter activity was measured using the Dual-Luciferase Reporter Assay System. In addition, IPEC-J2 cells were transfected with a control or plasmid for RXR-α, PXR, VDR or CDR over-expression for 18 h and treated with, or without, 108 CFU/mL HDRsEf1 for 6 h. The impact of RXR-α, PXR, VDR or CDR over-expression on CYP3A29 expression was determined.

RNA Interference

IPEC-J2 cells were cultured in 12-well plates overnight and transfected with 40 nM control scramble or gene-specific siRNA using Lipofectamine 2000 for 36 h. The cells were treated with, or without, 108 CFU/mL HDRsEf1 for 6 h. The efficacy of specific gene silencing and its impact on the expression of other genes were quantified by qRT-PCR and Western blot.

Co-immunoprecipitation (Co-IP)

The physical association between PXR and RXR-α was characterized by Co-IP using SureBeads™ Starter Kit Protein G (Bio-Rad) (Xie et al., 2019). Briefly, RXR-α, PXR and specific antibody IgG or control isotype IgG (10 μg each) were reacted and the formed immunocomplex was precipitated with the microbeads, followed by eluting the proteins with laemmli buffer for subsequent Western blot analysis.

Statistical analysis

Data are present as mean ± S.D. Comparison was performed by Student's t-test, one-way ANOVA, or two-way ANOVA. Significant difference was defined when a P-value of < 0.05.

Results

HDRsEf1 reduces CYP3A29 expression *in vivo* and *in vitro*

Previous reports have shown that probiotics can regulate the expression of CYP450s in the intestinal tract of animals (Jourova et al., 2017; Kato et al., 2007). Because CYP3A29 is an important enzyme in the intestine of pigs, we examined whether HDRsEf1 could modulate the CYP3A29 expression in pig intestinal tissues. After fed with HDRsEf1-contained diet for 30 days, we found that the relative levels of CYP3A29 expression in the jejunum tissues of the probiotic group of pigs were significantly lower than that in the control group (Fig, 1A). Furthermore, treatment with 10^8 CFU/mL of HDRsEf1 for 6 h did not alter the viability of IPEC-J2, but treatment with HDRsEf1 for a longer time period obviously decreased the viability of IPEC-J2 cells (Fig. 1B). In addition, treatment with 10^6, 10^7 and 10^8 CFU/mL of HDRsEf1for 2-6 h down-regulated CYP3A29 mRNA transcripts in IPEC-J2 cells in a dose- and time-dependent manner (Fig. 1C). Moreover, treatment with 10^8 CFU/mL of HDRsEf1 for 2-6 h in a transwell system revealed that HDRsEf1 inhibited CYP3A29 expression in IPEC-J2 cells only when IPEC-J2 cells were co-cultured with HDRsEf1 in the same wells, but not in the separated transwells. Hence, HDRsEf1 down-regulated the expression of intestinal CYP3A29 *in vivo* and *in vitro,* dependent on cell-cell contact.

HDRsEf1 down-regulates the expression of CYP3A29 at the transcriptional level

To investigate the effect of HDRsEf1 on the transcription of CYP3A29, we performed a transcriptional inhibition assay. We found that treatment with DRB, an inhibitor of RNA synthesis, completely abolished the HDRsEf1-down-regulated CYP3A29 transcription in IPEC-J2 cells (Fig. 2A). We further cloned the potential sequences of the CYP3A29 promoter of *Sus scrofa* into the pGL3-BASIC (Fig. 2B). After transfection, we performed luciferase reporter assays and found that all sequences exhibited varying levels of promoter activities and the highest levels of promoter activity were from the pGL3-3A29-371-transfected cells (Fig. 2B). More importantly, HDRsEf1 treatment significantly mitigated the pGL3-3A29-371 and pGL3-3A29-2007 (the longest sequence)-control luciferase expression following co-culture with IPEC-J2 cells, but not in separated transwell culture (Fig. 2C and D). Together, such data indicated that HDRsEf1 inhibited the transcription activity of the CYP3A29 promoter, dependent on cell-cell contact.

HDRsEf1 down-regulates CYP3A29 expression via RXR-α

NRs are crucial for the expression of CYPP450s(Honkakoski and Negishi, 2000). We found that HDRsEf1 treatment significantly decreased the relative levels of RXR-α, but not PXR, CAR and VDR mRNA

transcripts and protein expression (Fig. 3A) and particularly reduced nuclear RXR-α protein levels in IPEC-J2 cells (Fig. 3B). Given that RXR-α often interacts with PXR, VDR and CAR to form heterodimers and regulate the expression of CYP450s (Lefebvre et al., 2010) we further tested whether altered their expression could modulate the HDRsEf1-inhibited CYP3A29 expression in IPEC-J2 cells. We found that RXR-α or PXR over-expression increased CYP3A29 expression while HDRsEf1 treatment abrogated or significantly reduced RXR-α, PXR and CYP3A29 expression in IPEC-J2 cells (Fig. 3C and 3D). In contrast, RXR-α or PXR silencing by specific siRNA also significantly decreased the relative levels of CYP3A29 expression in IPEC-J2 cells (Fig. 3E and 3F). However, altered VDR or CAR expression did not affect the expression of CYP3A29 in IPEC-J2 cells (Fig. S1). Interestingly, both RXR-α and PXR over-expression further significantly increased the activity of pGL-3A29-2007 (Fig. 3G) and CYP3A29 protein expression (Fig. 3H), compared to RXR-α or PXR over-expression in IPEC-J2 cells. Co-IP assay revealed that RXR-α directly interacted with PXR in IPEC-J2 cells (Fig. 3I). Collectively, HDRsEf1 down-regulated CYP3A29 expression, dependent on inhibiting RXR-α expression and its interaction with PXR in IPEC-J2 cells.

HDRsEf1 down-regulates RXR-α expression by attenuating the NF-κB signaling

The NF-κB activation can significantly up-regulate the expression of RXR-α (Ren et al., 2016) while it can be inhibited by some probiotics (Finamore et al., 2014; Kawano et al., 2019). Therefore, we speculated that the down-regulated RXR-α expression by HDRsEf1 may stem from its down-regulation on the NF-κB activation. We found that HDRsEf1 treatment significantly decreased the NF-κ Bp65-driven luciferase expression in IPEC-J2 cells (Fig. 4A). Furthermore, HDRsEf1 treatment significantly reduced NF-kBp65, RXR-α and CYP3A29 expression and NF-kBp65 phosphorylation in IPEC-J2 cells (Fig. 4B-D). A similar pattern of inhibition on RXR-α and CYP3A29 expression and NF-kB activation was achieved by treatment with BAY-7082, an inhibitor of NF-kB, in IPEC-J2 cells (Fig. 4B and 4D). In addition, although the over-expressed NF-kBp65 masked endogenous NF-kBp65 detection in an automated imaging condition the NF-kBp65 over-expression significantly increased RXR-α and CYP3A29 expression, which were significantly mitigated by HDRsEf1 treatment in IPEC-J2 cells (Fig. 4E and 4F). Interestingly, HDRsEf1 failed to modulate significantly the relative levels of CYP3A29 expression in the RXR-α-silenced IPEC-J2 cells (Fig. 4G and 4H). Thus, HDRsEf1 attenuated the NF-kBp65 activation to inhibit the CYP3A29 and RXR-α expression in IPEC-J2 cells.

HDRsEf1 induces A20 to inhibit CYP3A29 expression, dependent on TLR1/2

Engagement of TLRs by Gram-positive bacteria can regulate the NF-κB activity in animal intestinal tissues (Bron et al., 2017). Finally, we examined whether and how any of the TLRs participated in the HDRsEf1-decreased CYP3A29 expression in IPEC-J2 cells. We detected TLR1, 2, 3, 4, 5 and 6 gene mRNA transcripts, which were effectively silenced by their specific siRNA in IPEC-J2 cells (Fig. 5A). HDRsEf1 treatment significantly decreased CYP3A29 mRNA transcripts in the TLR3, 4, 5 and 6-silenced and wild-

type IPEC-J2 cells, but not in the TLR1 or 2-silenced cells (Fig. 5B). Such data suggest that the down-regulated CYP3A29 expression by HDRsEf1 may depend on the sufficient expression of TLR1/2 in IPEC-J2 cells.

Given that engagement of TLRs by some Grams-positive bacteria can enhance the expression of some NF-κB inhibitors, attenuating the NF-kB activation (Finamore et al., 2014) we further determined the effect of HDRsEf1 on the expression of A20, IRAK-M and Tollip in IPEC-J2 cells. We found that HDRsEf1 treatment significantly increased A20, but not IRAK-M and Tollip, mRNA transcripts and protein expression in IPEC-J2 cells (Fig. 5C). The enhanced A20 mRNA transcripts by HDRsEf1 were abrogated by TLR1 or TLR2 silencing in IPEC-J2 cells, indicating that HDRsEf1 increased A20 expression in a TLR1/2-dependent manner (Fig. 5D). Actually, A20 silencing by specific siRNA increased CYP3A29 and RXR-α expression and abrogated the HDRsEf1-reduced CYP3A29 and RXR-α expression in IPEC-J2 cells (Fig. 4E and 4F). In contrast, A20 over-expression decreased CYP3A29 and RXR-α expression (Fig. 4E and 4F). Therefore, HDRsEf1 up-regulated A20 to down-regulate CYP3A29 expression in IPEC-J2 cells in a TLR1/2-dependent manner.

Discussion

Although many people believe that probiotics are highly safe for animals and humans a few people understand the effects of probiotics on the expression of DMEs in the intestine due to intestinal first-pass metabolism. Actually, probiotics can modulate the expression of intestinal DMEs (Jourova et al., 2017; Kato et al., 2007), but the mechanisms underlying the action of probiotics remain unclear. Our results indicated that HDRsEf1 down-regulated the expression of CYP3A29 in pig intestinal tissues through the TLR1/2-induced A20 expression to attenuate the NF-κB/RXRα pathway.

TLRs can recognize bacterial components (Iwasaki and Medzhitov, 2004). TLR2 can interact with TLR1 or TLR6 to form a heterodimer (Jin et al., 2007), and recognize Gram-positive bacterial cell wall components, such as peptidoglycan and lipoteichoic acid (Asong et al., 2009; Ghose et al., 2009). In this study, we found that HDRsEf1 enhanced A20 expression in pig intestinal epithelia cells, dependent on TLR1/2. Given that A20 is an inhibitor of the NF-kB signalling by degrading TRAF-6(Shembade et al., 2010) the enhanced A20 expression by HDRsEf1 should contribute to its inhibition on the NF-kBp65 activation and CYP3A29 expression in IPEC-J2 cells. In fact, we found that HDRsEf1, like the NF-kB inhibitor of BAY11-7082, significantly attenuated the NF-kB activation and RXR-α expression in IPEC-J2 cells. Such data extended previous observations that probiotics, such as Lactobacillus amylovorus and paracasei, can bind to TLR2 to regulate the expression of NF-κB inhibitors, including A20, SOCS1 and SOCS3 in macrophages (Kawano et al., 2019)·(Sun et al., 2017) and support the notion that Gram-positive bacteria can negatively regulate inflammation through the TLR1/2 to induce the expression of the NF-kB inhibitors (Finamore et al., 2014; Ren et al., 2016).

Furthermore, we found that HDRsEf1 significantly reduced the expression of RXR-α and CYP3A29 in IPEC-J2 cells, which were abrogated by NF-kBp65 overexpression, suggesting that the enhanced NF-kBp65

activity may up-regulate RXR-α expression and subsequent CYP3A29 expression. These, together with the fact that HDRsEf1 failed to decrease CYP3A29 expression in the RXR-α-silenced IPEC-J2 cells, indicated that the NF-κBp65 signaling enhanced the CYP3A29 expression indirectly by enhancing RXR-α expression in intestinal tissues. Previous reports indicate that Staphylococcus aureus-derived LTA can reduce RXR-α expression in mouse liver by inhibiting the JNK and NF-κB pathways (Ghose et al., 2009), while IL-1β enhances RXR-α expression by activating the NF-kB signaling in gastric carcinoma tissues (Ren et al., 2016). Apparently, the NF-kB signaling positively regulates the expression of RXRα, which can attenuate the inhibition of NF-kB activation (Ning et al., 2013), maintaining the balance of intracellular inflammatory and anti-inflammatory responses. Interestingly, we found that HDRsEf1 did not modulate PXR expression in IPEC-J2 cells. These data were in disagreement with previous reports that the phosphorylated NF-κBp65 can down-regulate PXR expression and inhibit the formation of PXR/RXR-α heterodimers and CYP3A4 transcription in HepG2 cells (Gu et al., 2006). The discrepancy suggests that the activated NF-κBp65 may have different regulatory roles in the expression of NRs in different types of cells. Given that the activated NF-κB usually regulates the transcription of the NRs (Zhou et al., 2016)，(Vogel et al., 2014) we are interested in further investigating how the activated NF-κBp65 regulates RXR-α expression in pig intestinal tissues.

In this study, we found that HDRsEf1 significantly reduced RXRα expression, particularly for nuclear RXRα protein levels, but did not affect the expression of other NRs tested in IPEC-J2 cells. Furthermore, RXRα or PXR overexpression and silencing significantly modulated CYP3A29 expression in IPEC-J2 cells and both RXR-α and PXR over-expression synergistically enhanced the CYP3A29 promoter activity. In addition, RXR-α directly interacted with PXR in IPEC-J2 cells. Together, such data suggest that RXR-α and PXR may form heterodimer for the CYP3A29 transcription in IPEC-J2 cells, consistent with our findings in HepLi cells(Zhou et al., 2016). However, we found that altered VDR or CAR expression did not affect the CYP3A29 expression in IPEC-J2 cells, implicating that the VDR/RXR-α and CAR/RXR-α might not be involved in up-regulating CYP3A29 expression although they are crucial for CYP3A4 transcription in human intestinal cells and HepG2(Chen et al., 2010; Pavek et al., 2010). Therefore, the NRs may have interspecies difference in regulating the transcription of CYP3As even if there are organ variants in the same species.

We found that HDRsEf1 down-regulated the expression of CYP3A29 in IPEC-J2 in a time- and dose-dependent manner. Although HDRsEf1 supernatants have been reported to mitigate the ETEC K88ac-up-regulated IL-8 expression(Tian et al., 2016), we found that HDRsEf1 inhibited the CYP3A20 expression in IPEC-J2 cells, dependent on cell-cell contact. Our findings were consistent with a recent report that Lactobacillus helveticus SBT2171 cell wall components inhibit inflammatory responses in peritoneal macrophage (Kawano et al., 2019). The difference between our and those with ETEC K88ac may stem from the presence of more pathogen-associated molecular patterns on the cell wall of Gram-positive bacteria. We will continually explore which cell wall components of HDRsEf1 can bind to TLR1/2 on intestinal epithelial cells to inhibit CYP3A29 expression.

Conclusion

Our data indicated that HDRsEf1 inhibited the CYP3A29 expression in pig intestinal tissues and IPEC-J2 cells in a dose-, time- and cell-cell contact-dependent manner. HDRsEf1 through TLR1/2-induced A20 expression attenuated the NF-kBp65 activation to reduce RXR-α expression and limit the formation of RXR-α/PXR heterodimer, inhibiting CYP3A29 transcription in intestinal epithelial cells (Fig. 7). Our findings may provide new insights into the mechanisms by which probiotics regulate the expression of CYP450s and highlight the possible risks of probiotics for oral drug overdose in the clinic.

Declarations

Acknowledgements

Not applicable

Funding

This work was supported by the National Key Research and Development Program of China (2017YFD0501000) and the Fundamental Research Funds for Central Universities (2662019PY061).

Author contributions

Y.H., D.S. and X.W. designed the research. Y.H., Y.L., Y.G., and X.J. performed the research. Y.H., Y.W., and X.W. analyzed the data. Y.H. and X.W. wrote the paper with the help of all authors. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Consent for publication

Not applicable

Ethics approval and consent to participate

See ethics paragraph in the “Materials and methods” section

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files]

References

Anzenbacher, P., Soucek, P., Anzenbacherova, E., Gut, I., Hruby, K., Svoboda, Z., and Kvetina, J. (1998). Presence and activity of cytochrome P450 isoforms in minipig liver microsomes. Comparison with human liver samples. Drug metabolism and disposition: the biological fate of chemicals *26*, 56-59.

Asong, J., Wolfert, M.A., Maiti, K.K., Miller, D., and Boons, G.J. (2009). Binding and Cellular Activation Studies Reveal That Toll-like Receptor 2 Can Differentially Recognize Peptidoglycan from Gram-positive and Gram-negative Bacteria. The Journal of biological chemistry *284*, 8643-8653.

Boone, D.L., Turer, E.E., Lee, E.G., Ahmad, R.C., Wheeler, M.T., Tsui, C., Hurley, P., Chien, M., Chai, S., Hitotsumatsu, O.*, et al.* (2004). The ubiquitin-modifying enzyme A20 is required for termination of Toll-like receptor responses. Nature immunology *5*, 1052-1060.

Bron, P.A., Kleerebezem, M., Brummer, R.J., and Cani, P.D. (2017). Can probiotics modulate human disease by impacting intestinal barrier function? *117*, 93-107.

Chen, S., Wang, K., and Wan, Y.J. (2010). Retinoids activate RXR/CAR-mediated pathway and induce CYP3A. Biochemical pharmacology *79*, 270-276.

Finamore, A., Roselli, M., Imbinto, A., Seeboth, J., Oswald, I.P., and Mengheri, E. (2014). Lactobacillus amylovorus inhibits the TLR4 inflammatory signaling triggered by enterotoxigenic Escherichia coli via modulation of the negative regulators and involvement of TLR2 in intestinal Caco-2 cells and pig explants. PloS one *9*, e94891.

Ghose, R., Guo, T., and Haque, N. (2009). Regulation of gene expression of hepatic drug metabolizing enzymes and transporters by the Toll-like receptor 2 ligand, lipoteichoic acid. Archives of biochemistry and biophysics *481*, 123-130.

Gu, X., Ke, S., Liu, D., Sheng, T., Thomas, P.E., Rabson, A.B., Gallo, M.A., Xie, W., and Tian, Y. (2006). Role of NF-kappaB in regulation of PXR-mediated gene expression: a mechanism for the suppression of cytochrome P-450 3A4 by proinflammatory agents. The Journal of biological chemistry *281*, 17882-17889.

Guengerich, F.P. (1997). Comparisons of catalytic selectivity of cytochrome P450 subfamily enzymes from different species. Chemico-biological interactions *106*, 161-182.

He, Y., Zhou, X., Li, X., Jin, X., Wang, X., Pan, X., and Bi, D. (2018). Relationship between CYP3A29 and pregnane X receptor in landrace pigs: Pig CYP3A29 has a similar mechanism of regulation to human CYP3A4. Comparative biochemistry and physiology Toxicology & pharmacology : CBP *214*, 9-16.

Honkakoski, P., and Negishi, M. (2000). Regulation of cytochrome P450 (CYP) genes by nuclear receptors. The Biochemical journal *347*, 321-337.

Iwasaki, A., and Medzhitov, R. (2004). Toll-like receptor control of the adaptive immune responses. Nature immunology *5*, 987-995.

Jin, M.S., Kim, S.E., Heo, J.Y., Lee, M.E., Kim, H.M., Paik, S.G., Lee, H., and Lee, J.O. (2007). Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. Cell *130*, 1071-1082.

Jourova, L., Anzenbacher, P., Liskova, B., Matuskova, Z., Hermanova, P., Hudcovic, T., Kozakova, H., Hrncirova, L., and Anzenbacherova, E. (2017). Colonization by non-pathogenic bacteria alters mRNA expression of cytochromes P450 in originally germ-free mice. Folia microbiologica *62*, 463-469.

Kato, R., Yuasa, H., Inoue, K., Iwao, T., Tanaka, K., Ooi, K., and Hayashi, Y. (2007). Effect of Lactobacillus casei on the absorption of nifedipine from rat small intestine. Drug metabolism and pharmacokinetics *22*, 96-102.

Kawano, M., Miyoshi, M., and Miyazaki, T. (2019). Lactobacillus helveticus SBT2171 Induces A20 Expression via Toll-Like Receptor 2 Signaling and Inhibits the Lipopolysaccharide-Induced Activation of Nuclear Factor-kappa B and Mitogen-Activated Protein Kinases in Peritoneal Macrophages. Frontiers in immunology *10*, 845.

Lee, S.H., Wang, X., and DeJong, J. (2000). Functional interactions between an atypical NF-kappaB site from the rat CYP2B1 promoter and the transcriptional repressor RBP-Jkappa/CBF1. Nucleic acids research *28*, 2091-2098.

Lefebvre, P., Benomar, Y., and Staels, B. (2010). Retinoid X receptors: common heterodimerization partners with distinct functions. Trends in endocrinology and metabolism: TEM *21*, 676-683.

Marchesi, J.R., Adams, D.H., Fava, F., Hermes, G.D., Hirschfield, G.M., Hold, G., Quraishi, M.N., Kinross, J., Smidt, H., Tuohy, K.M.*, et al.* (2016). The gut microbiota and host health: a new clinical frontier. Gut *65*, 330-339.

Ning, R.B., Zhu, J., Chai, D.J., Xu, C.S., Xie, H., Lin, X.Y., Zeng, J.Z., and Lin, J.X. (2013). RXR agonists inhibit high glucose-induced upregulation of inflammation by suppressing activation of the NADPH oxidase-nuclear factor-kappaB pathway in human endothelial cells. Genetics and molecular research : GMR *12*, 6692-6707.

Pavek, P., Pospechova, K., Svecova, L., Syrova, Z., Stejskalova, L., Blazkova, J., Dvorak, Z., and Blahos, J. (2010). Intestinal cell-specific vitamin D receptor (VDR)-mediated transcriptional regulation of CYP3A4 gene. Biochemical pharmacology *79*, 277-287.

Pereira, S.G., and Oakley, F. (2008). Nuclear factor-kappaB1: regulation and function. The international journal of biochemistry & cell biology *40*, 1425-1430.

Ren, H.Y., Huang, G.L., Liu, W.M., Zhang, W., Liu, Y., Su, G.Q., and Shen, D.Y. (2016). IL-1beta induced RXRalpha overexpression through activation of NF-kappaB signaling in gastric carcinoma. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie *78*, 329-334.

Rodriguez-Antona, C., Gomez, A., Karlgren, M., Sim, S.C., and Ingelman-Sundberg, M. (2010). Molecular genetics and epigenetics of the cytochrome P450 gene family and its relevance for cancer risk and treatment. Human genetics *127*, 1-17.

Shang, H., Guo, K., Liu, Y., Yang, J., and Wei, H. (2013). Constitutive expression of CYP3A mRNA in Bama miniature pig tissues. Gene *524*, 261-267.

Shembade, N., Ma, A., and Harhaj, E.W. (2010). Inhibition of NF-kappaB signaling by A20 through disruption of ubiquitin enzyme complexes. Science (New York, NY) *327*, 1135-1139.

Skaanild, M.T. (2006). Porcine cytochrome P450 and metabolism. Current pharmaceutical design *12*, 1421-1427.

Sun, K.Y., Xu, D.H., Xie, C., Plummer, S., Tang, J., Yang, X.F., and Ji, X.H. (2017). Lactobacillus paracasei modulates LPS-induced inflammatory cytokine release by monocyte-macrophages via the up-regulation of negative regulators of NF-kappaB signaling in a TLR2-dependent manner. Cytokine *92*, 1-11.

Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K., and Akira, S. (1999). Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity *11*, 443-451.

Tian, Z., Liu, X., Dai, R., Xiao, Y., Wang, X., Bi, D., and Shi, D. (2016). Enterococcus faecium HDRsEf1 Protects the Intestinal Epithelium and Attenuates ETEC-Induced IL-8 Secretion in Enterocytes. *2016*, 7474306.

Toriyabe, T., Nagata, K., Takada, T., Aratsu, Y., Matsubara, T., Yoshinari, K., and Yamazoe, Y. (2009). Unveiling a new essential cis element for the transactivation of the CYP3A4 gene by xenobiotics. Molecular pharmacology *75*, 677-684.

van Herwaarden, A.E., van Waterschoot, R.A., and Schinkel, A.H. (2009). How important is intestinal cytochrome P450 3A metabolism? Trends in pharmacological sciences *30*, 223-227.

van Waterschoot, R.A., Rooswinkel, R.W., Wagenaar, E., van der Kruijssen, C.M., van Herwaarden, A.E., and Schinkel, A.H. (2009). Intestinal cytochrome P450 3A plays an important role in the regulation of detoxifying systems in the liver. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *23*, 224-231.

Vogel, C.F., Khan, E.M., Leung, P.S., Gershwin, M.E., Chang, W.L., Wu, D., Haarmann-Stemmann, T., Hoffmann, A., and Denison, M.S. (2014). Cross-talk between aryl hydrocarbon receptor and the inflammatory response: a role for nuclear factor-kappaB. The Journal of biological chemistry *289*, 1866-1875.

Wu, J., Chen, R., Zhang, C., Li, K., Xu, W., Wang, L., Chen, Q., Mu, P., Jiang, J., Wen, J.*, et al.* (2016). Bioactivation and Regioselectivity of Pig Cytochrome P450 3A29 towards Aflatoxin B(1). Toxins *8*.

Yan, T., Zhang, F., He, Y., and Wang, X. (2018). Enterococcus faecium HDRsEf1 elevates the intestinal barrier defense against enterotoxigenic Escherichia coli and regulates occludin expression via activation of TLR-2 and PI3K signalling pathways. *67*, 520-527.

Zangar, R.C., Bollinger, N., Verma, S., Karin, N.J., and Lu, Y. (2008). The nuclear factor-kappa B pathway regulates cytochrome P450 3A4 protein stability. Molecular pharmacology *73*, 1652-1658.

Zhou, X., Li, X., Wang, X., Jin, X., Shi, D., Wang, J., and Bi, D. (2016). Cecropin B Represses CYP3A29 Expression through Activation of the TLR2/4-NF-kappaB/PXR Signaling Pathway. Scientific reports *6*, 27876.

Zordoky, B.N., and El-Kadi, A.O. (2009). Role of NF-kappaB in the regulation of cytochrome P450 enzymes. Current drug metabolism *10*, 164-178.

Figures

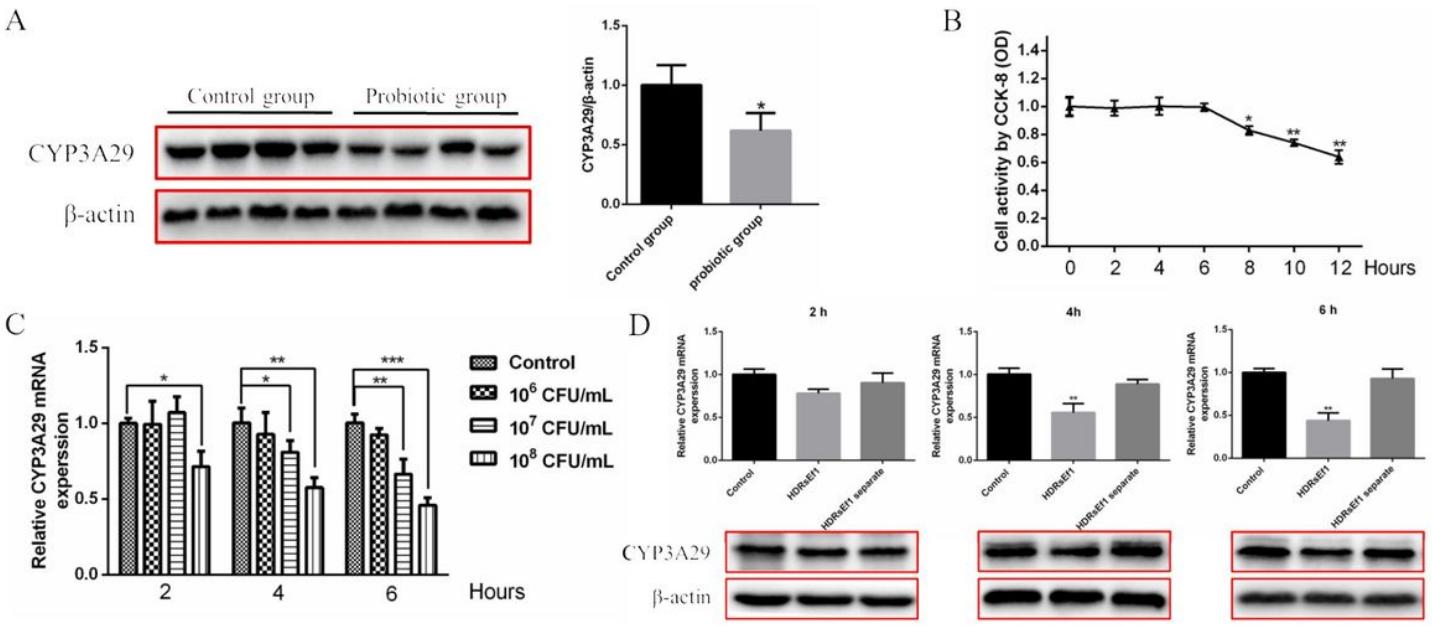

Figure 1

HDRsEf1 reduces CYP3A29 gene expression in vivo and in vitro. (A) Western blot analysis of CYP3A29 protein expression in pig jejunum tissues (n = 4 per group). (B) CCK-8 assay analysis of IPEC-J2 cell viability after treatment with 108 CFU/mL of HDRsEf1 for 2, 4, 6, 8, 10 and 12 h. (C) Quantitative RT-PCR analysis of CYP3A29 mRNA transcripts in IPEC-J2 cells after treatment with the indicated doses of HDRsEf1 for varying periods. (D) HDRsEf1 decreased CYP3A29 expression in IPEC-J2 cells in a cell-cell

contact-dependent manner. IPEC-J2 cells were co-cultured with 108 CFU/mL of HDRsEf1 in the same wells or separately cultured in transwell plates (separate) for the indicated time periods. The relative levels of CYP3A29 mRNA transcripts and protein expression in individual groups of cells were quantified by qRT-PCR and Western blotting. Data are representative images or expressed as the mean ± SD of each group from three separate experiments. *p<0.05; **p<0.01; ***p<0.001.

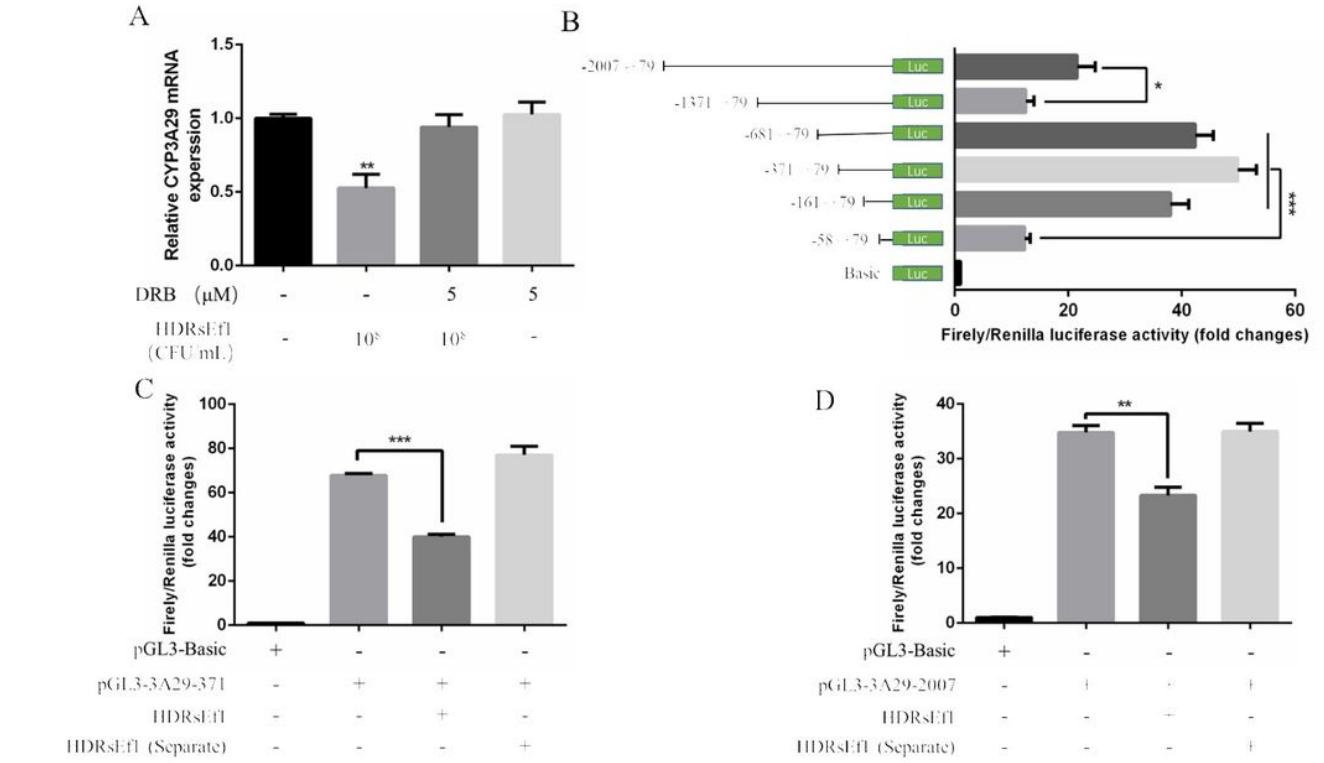

Figure 2

HDRsEf1 down-regulates the CYP3A29 transcription in IPEC-J2 cells. (A) IPEC-J2 cells were treated with 108 CFU/mL of HDRsEf1 for 6 h in the absence or presence of 5 μM DRB and the relative levels of CYP3A29 mRNA transcripts were quantified by qRT-PCR. (B) IPEC-J2 cells were transiently transfected with the indicated plasmids containing the sequences of the CYP3A29 promoter and one day later, their promoter activities were determined by dual luciferase reporter assays. (C, D) IPEC-J2 cells were transiently transfected with pGL3-3A29-371 (C) and pGL3-3A29-2007 (D), and 18 h later, the transfected cells were co-cultured or separated culture with 108 CFU/mL of HDRsEf1 for 6 h, followed by detecting luciferase activity. Data are expressed as the mean ± SD of each group from three separate experiments. *p<0.05; **p<0.01; ***p<0.001.

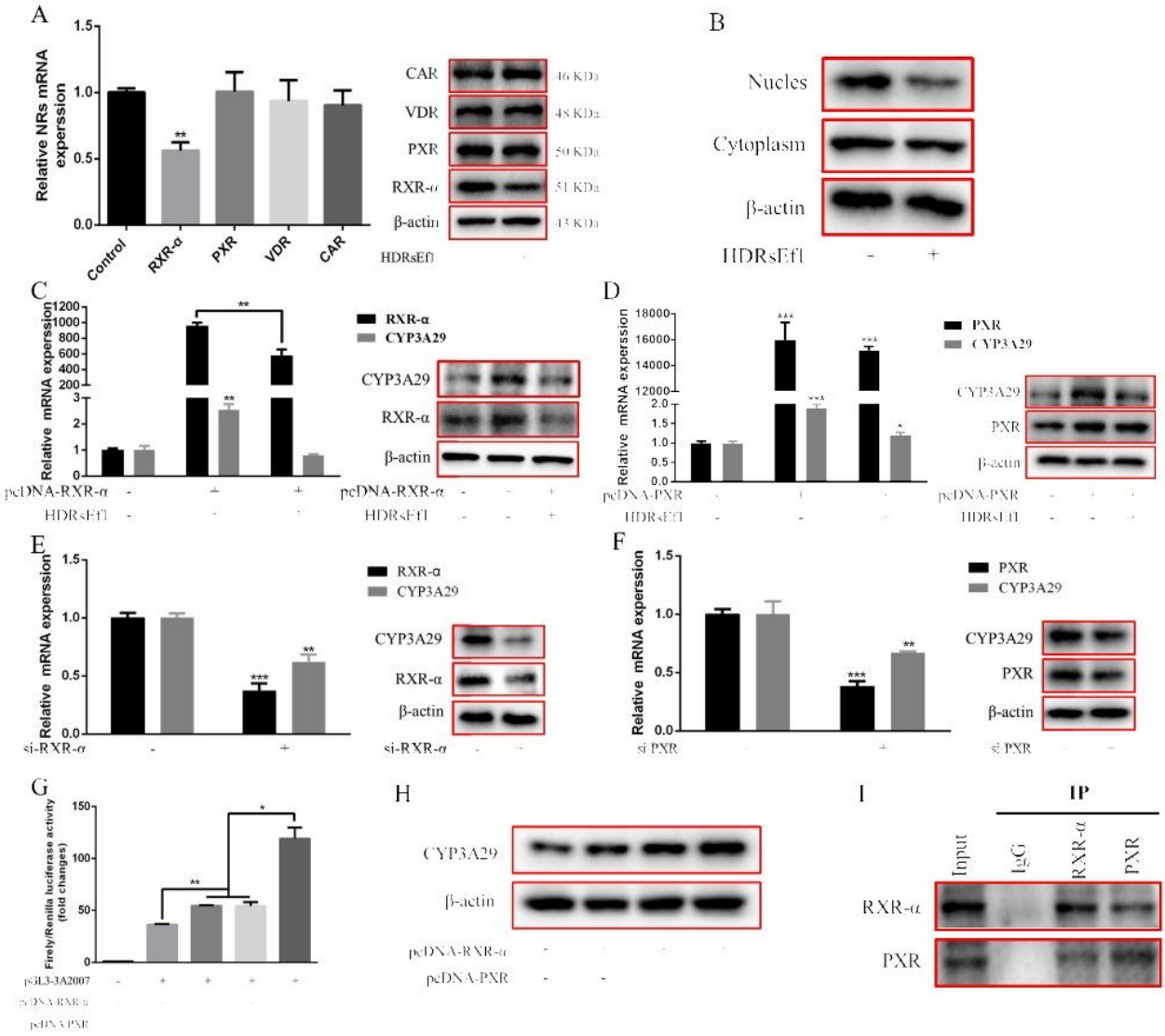

Figure 3

HDRsEf1 down-regulates CYP3A29 expression by reducing RXR-α expression in IPEC-J2 cells. (A) IPEC-J2 cells were treated with 108 CFU/mL of HDRsEf1 for 6 h, and the relative levels of RXR-α, PXR, VDR and CAR mRNA transcripts and protein expression were quantified by qRT-PCR and Western blotting. (B) IPEC-J2 cells were treated with 108 CFU/mL of HDRsEf1 for 6 h and their cytoplasmic and nuclear levels of RXR-α were quantified by Western blotting. (C, D) IPEC-J2 cells were transfected with plasmid for RXR-α (C) or PXR (D) over-expression or control vector for 18 h and treated with 108 CFU/mL of HDRsEf1 for 6 h. The relative levels of CYP3A29, RXR-α or PXR mRNA transcripts and protein expression were quantified by qRT-PCR and Western blotting. (E, F) IPEC-J2 cells were transfected with control or RXR-α-specific (E) or PXR-specific siRNA (F) for 36 h. The relative levels of CYP3A29, RXR-α or PXR mRNA transcripts and protein expression were quantified by qRT-PCR and Western blotting. (G) IPEC-J2 cells were co-transfected with the pGL3-3A29-2007, the plasmid for Renilla luciferase expression, and pcDNA-RXR-α and/or pcDNA-PXR. The luciferase activity in each group of cells was determined by dual luciferase reporter assays. (H) Co-IP analysis of RXR-α and PXR interaction. Data are representative images or

expressed as the mean ± SD of each group from three separate experiments. *p<0.05; **p<0.01; ***p<0.001.

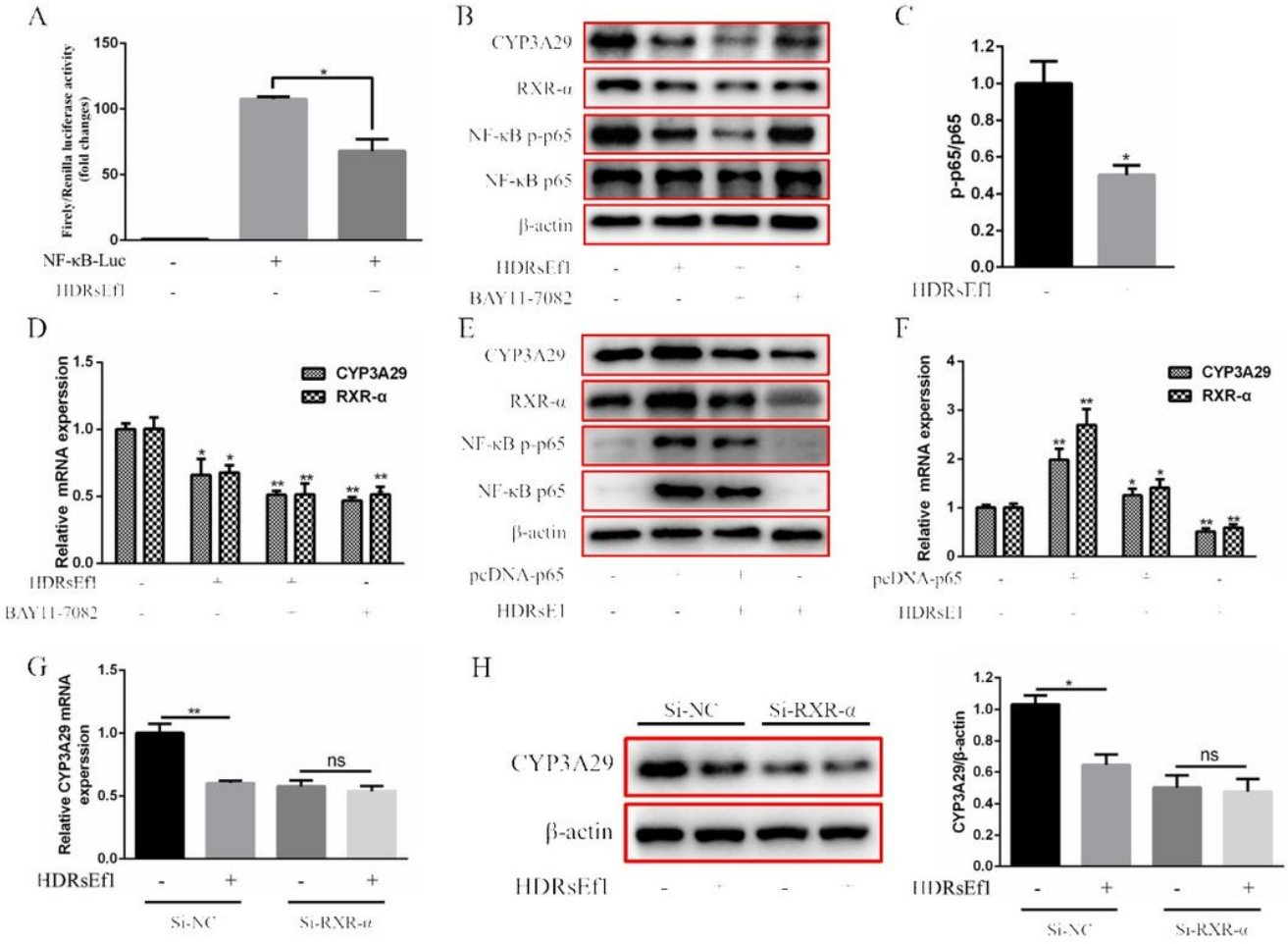

Figure 4

HDRsEf1 down-regulates RXR-α expression by inhibiting the NF-κB activation. (A) IPEC-J2 cells were transfected with the plasmids containing the NF-κB promoter-driven luciferase and Renilla luciferase expression for 24 h. The cells were treated with, or without, 108 CFU/mL of HDRsEf1, and 6 h later, their luciferase activity was examined. (B, D) IPEC-J2 cells were treated with 108 CFU/mL of HDRsEf1 and/or 10 μM BAY11-7082 for 6 h, and the relative levels of CYP3A29, RXR-α, NF-kBp65 expression and NF-kBp65 phosphorylation were quantified by Western blotting (B) and qRT-PCR (D). (C) The ratios of phosphorylated NF-kBp65 to NF-kBp65. (E, F) IPEC-J2 cells were transfected with the plasmid for NF-κBp65 overexpression or control vector for 18 h, and treated with, or without, 108 CFU/mL of HDRsEf1 for 6 h. The relative levels of CYP3A29, RXR-α, NF-kBp65 expression and NF-kBp-p65 phosphorylation were quantified by Western blotting (E) and qRT-PCR (F). (G, H) IPEC-J2 cells were transfected with 20 μM control or RXR-α-specific siRNA for 30 h, and treated with, or without, 108 CFU/mL of HDRsEf1 for 6 h. The relative levels of CYP3A29 expression were quantified by qRT-PCR and Western blotting. Data are representative images or expressed as the mean ± SD of each group from three separate experiments. *p<0.05; **p<0.01; ***p<0.001.

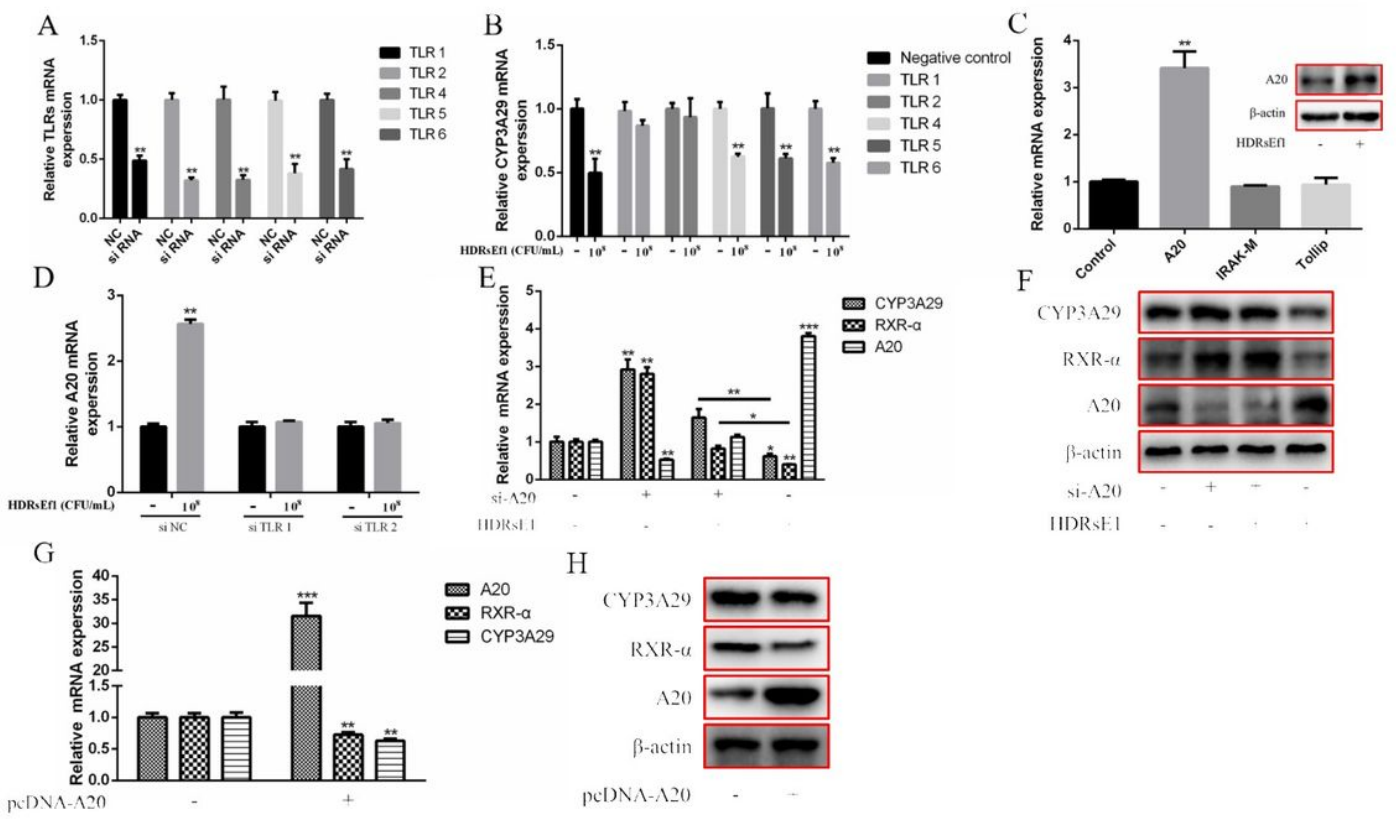

Figure 5

HDRsEf1 induces A20 and inhibits CYP3A29 expression in IPEC-J2 cells, dependent on TLR1/2. (A) IPEC-J2 cells were transfected with control or TLR 1/2/4/5/6 specific siRNA for 36 h. The RNA interference efficiency was determined by qRT-PCR. (B) IPEC-J2 cells were transfected with control or TLR1/2/4/5/6 specific siRNA for 30 h, and treated with 108 CFU/mL of HDRsEf1 for 6 h. The relative levels of CYP3A29 mRNA transcripts were quantified by qRT-PCR. (C) IPEC-J2 cells were treated with 108 CFU/mL of HDRsEf1 for 6 h and the relative levels of A20, IRAK-M and Tollip mRNA transcripts were quantified by qRT-PCR. (D) IPEC-J2 cells were transfected with control or TLR1/2 specific siRNAs for 30 h, and treated with, or without, 108 CFU/mL of HDRsEf1 for 6 h. The relative levels of CYP3A29 mRNA transcripts were quantified by qRT-PCR. (E, F) IPEC-J2 cells were transfected with 20 μM control or A20-specific siRNA for 30 h, and treated with, or without, 108 CFU/mL of HDRsEf1 for 6 h. The relative levels of CYP3A29 expression were quantified by qRT-PCR and Western blotting. (E, F) IPEC-J2 cells were transfected with the plasmid for A20 over-expression or control vector for 24 h. The relative levels of CYP3A29, RXR-α and A20 expression were quantified by qRT-PCR and Western blotting. Data are expressed as the mean ± SD of each group from three separate experiments. *p<0.05; **p<0.01; ***p<0.001.

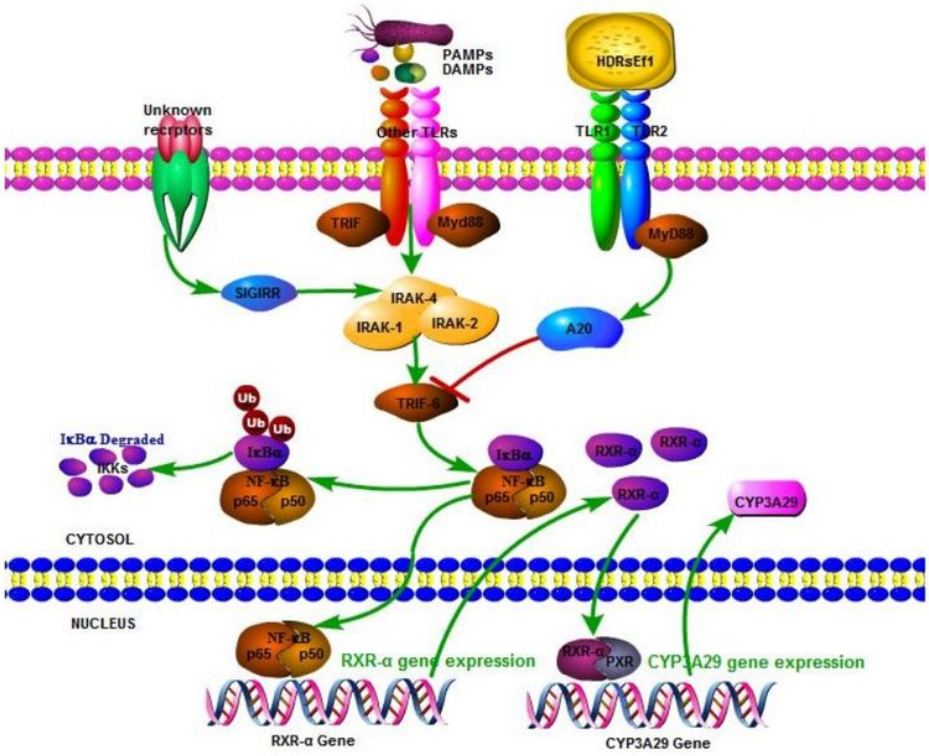

Figure 6

Schematic illustration of the possible mechanisms underlying the suppression of HDRsEf1 on CYP3A29 expression in the intestinal tissues. HDRsEf1 binds to TLR1/2 to up-regulate A20 expression, which inhibits the NF-κB activation by degrading TRIF6 and down-regulates RXR-α expression to reduce the RXR-α/PXR heterodimer formation and CYP3A29 expression.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.docx
- TableS2.docx
- Fig.S1.tif