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PPAR- γ AGONIST PROTECTS AGAINST INTESTINAL INJURY DURING NECROTIZING ENTEROCOLITIS

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Abstract

Necrotizing enterocolitis (NEC) remains a lethal condition for many premature infants. Peroxisome proliferator-activated receptor- γ (PPAR- γ), a member of the nuclear hormone receptor family, has been shown to play a protective role in cellular inflammatory responses; however, its role in NEC is not clearly defined. We sought to examine the expression of PPAR- γ in the intestine using an ischemia-reperfusion (I/R) model of NEC, and to assess whether PPAR- γ agonist treatment would ameliorate I/R-induced gut injury. Swiss-Webster mice were randomized to receive sham (control) or I/R injury to the gut induced by transient occlusion of superior mesenteric artery for 45 min with variable periods of reperfusion. I/R injury resulted in early induction of PPAR- γ expression and activation of NF- κ B in small intestine. Pretreatment with PPAR- γ agonist, 15d-PGJ₂, attenuated intestinal NF- κ B response and I/R-induced gut injury. Activation of PPAR- γ demonstrated a protective effect on small bowel during I/R-induced gut injury.

Keywords

Peroxisome proliferator-activated receptor- γ ; Nuclear factor-kappa B; Ischemia-reperfusion injury; Necrotizing enterocolitis

INTRODUCTION

Necrotizing enterocolitis (NEC), characterized by disruption of the mucosal barrier, transmural intestinal injury and significant inflammatory response typically involving terminal ileum and colon, is the most common gastrointestinal surgical emergency in premature neonates. It occurs in 5 to 10% of very-low-birth weight premature infants, resulting in significant morbidity and mortality [1]. Recently, the incidence of NEC has steadily increased as a result of improved survival of low-birth weight premature infants. Although a number of possible etiologies have been postulated, the exact pathogenesis of NEC remains poorly understood. A variety of contributing factors, such as ischemia/reperfusion (I/R) injury, hypoxia, reactive oxygen species (ROS), pro-inflammatory cytokines (TNF α , IL-1), have been identified in recent years [2;3; 4;5;6]; however, the exact cellular signaling pathways involved in intestinal injury during NEC still remain unclear.

NF- κ B is an important protein for the activation of many inflammatory mediators and cytokines [7]. The NF- κ B proteins, p50 and p65, are expressed in all cell types and are normally

sequestered in the cytoplasm bound to the inhibitory protein I κ B. Upon activation, I κ B is rapidly phosphorylated and degraded by proteasomes, allowing NF- κ B to translocate into the nucleus where it binds to its consensus sequence within the promoter region of various target genes. The activation of NF- κ B is involved in several inflammatory conditions, such as inflammatory bowel disease and pancreatitis. Previously, we demonstrated an induction of NF- κ B binding activity following ischemic injury in the small intestine of rats using an experimental model of NEC [4]. Recently, inhibition of I κ B phosphorylation using NBD peptide-affording selective inhibition of NF- κ B activity-was shown to decrease mortality and bowel injury in an animal NEC model [8]. Furthermore, activation/inhibition of upstream regulators of NF- κ B may offer an additional mechanism of protection from bowel injury in NEC.

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor supergene family that function through ligand-mediated transcription [9]. PPARs have been shown to play a key role in adipose differentiation, lipid metabolism, glucose homeostasis, and more recently, in the control of inflammation and cytokine modulation in monocytes and macrophages [10;11]. PPARs consist of 3 isoforms (α , β , γ) that are encoded by unique genes and distributed in different tissues. Studies focusing on cellular inflammatory responses indicate that PPAR- γ plays a protective role in various tissues [12;13;14]. PPAR- γ ligands demonstrate attenuation of the inflammatory response in colon, Alzheimer's disease, arthritis, and aspirin-induced gastric inflammation [15;16;17;18]. PPAR- γ has also been identified as an endogenous anti-inflammatory regulator in an intestinal I/R injury model [19]. The mechanism of the anti-inflammatory action of PPAR- γ ligands has partly been attributed to inhibition of the NF- κ B pathway. PPAR- γ has been shown to inhibit NF- κ B by associating with the p65 subunit in the nucleus and promoting export of the PPAR- γ :p65 complex to the cytosol [3]. However, the role of PPAR- γ in the regulation of NF- κ B activity and the effects of PPAR- γ agonists in NEC are unknown.

In this paper, we sought to further discern the molecular mechanisms of NEC by examining the potential role of PPAR- γ in the disease process. We investigated intestinal expression of PPAR- γ using *in vivo* NEC model in mice, and also examined the role of PPAR- γ in the regulation of NF- κ B during NEC using a high-affinity ligand for PPAR- γ .

MATERIALS AND METHODS

Reagents

Tissue culture media and reagents were obtained from Mediatech, Inc (Herndon, VA). TNF- α , hydrogen peroxide (H₂O₂), sterile normal saline solution, PBS, polyclonal anti-rabbit PPAR- γ antibody, and mouse monoclonal anti- β -actin antibody were purchased from Sigma (St. Louis, Mo). 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15d-PGJ₂), a PPAR- γ ligand, was obtained from Calbiochem (La Jolla, CA). Polyvinylidene difluoride (PVDF) membranes were from Millipore Corp. (Bedford, MA). Enhanced chemiluminescence (ECL)^{Plus} system was purchased from Amersham Biosciences (Piscataway, NJ).

Intestinal I/R animal model

All experimental protocols were approved by IACUC of the University of Texas Medical Branch (Galveston, TX). Adult Swiss-Webster mice were purchased from Charles River Laboratories (Pontage, MI), acclimated for one week, and then randomized into sham or I/R group. After anesthesia (pentobarbital; 40 mg/kg; ip), abdomen was opened at midline, and superior mesenteric artery (SMA) was transiently occluded for 45 min using non-traumatic vascular clamps, and then released. Reperfusion times ranged from 30 min to 3 h. Sham animals underwent an identical procedure without SMA occlusion. Mice received intra-peritoneal NS

fluid resuscitation (10 cc/kg). At sacrifice, small intestine was harvested for tissue and protein analysis. Segments of ileum and jejunum were harvested, fixed in formalin and stored in 70% ethanol for paraffin embedding. The remaining tissue was snap frozen in liquid nitrogen for protein analysis. Histological changes were assessed and scored by a pathologist in a blinded fashion.

Activation of PPAR- γ in I/R model of NEC

PPAR- γ protein expression was analyzed by Western immunoblotting. Tissue lysates prepared from mouse intestines were clarified by centrifugation ($13000 \times g$ for 20 min at 4°C) and protein concentrations were determined using the Bradford method. Equal amounts of total protein (100 μg) were loaded onto NUPAGE 4–12% Bis-Tris Gel and transferred to PVDF membranes, incubated in a blocking solution for 1 h (Tris-buffered saline containing 5% nonfat dried milk and 0.1 % Tween 20), and then incubated with primary antibody overnight at 4°C and horseradish peroxidase-conjugated secondary antibody. Anti- β -actin antibody was used for protein loading control. The immune complexes were visualized by ECL^{Plus}.

PPAR- γ ligand, 15d-PGJ₂, pretreatment during I/R injury

Adult Swiss-Webster mice were randomized to receive intraperitoneal (i.p.) injections of either high-affinity PPAR- γ ligand 15d-PGJ₂ (2 mg/kg) or vehicle (PBS) 45 min prior to IR injury. At sacrifice, jejunum and ileum were harvested and nuclear protein extracts (5 μg) were analyzed using electrophoretic mobility shift assays (EMSA) to determine the NF- κB binding activity. Segments of ileum and jejunum were fixed in formalin and stored in 70% ethanol for paraffin embedding. Tissue sections were cut into 5- μm sections and stained with hematoxylin and eosin and examined under light microscope. Histological changes were assessed by a pathologist and scored as previously described [20].

Cell culture

Human HT29 intestinal cells were obtained from ATCC and were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum (FBS). All cells were maintained at 37°C under an atmosphere containing 5% CO_2 . HT-29 cells (2×10^4) were plated 24 h prior to pretreatment with 15d-PGJ₂ (5–30 μM ; 30 min) followed by treatment with TNF- α (1 nM; 30 min). Nuclear protein extracts (5 μg) were obtained using a nuclear extraction kit (Pierce, Rockford, IL), and were added to a labeled oligonucleotide probe containing the consensus NF- κB binding site, and then resolved by gel mobility shift assay.

Western blot analysis

Mouse ileal and jejunal lysates were clarified with centrifugation (13200 rpm, 20 min at 4°C) and stored at -80°C . Protein concentrations were determined using the Bradford method. Equal amounts of total protein (100 μg) were loaded onto NUPAGE 4–12% Bis-Tris Gel and transferred to PVDF membranes, incubated in a blocking solution for 1 h (Tris-buffered saline containing 5% nonfat dried milk and 0.1 % Tween 20), incubated with PPAR- γ primary antibody overnight at 4°C , and then incubated with horseradish peroxidase-conjugated secondary antibody. Anti- β -actin antibody was used for protein loading control. The immune complexes were visualized by ECL^{Plus} (Amersham Biosciences, Piscataway, NJ). Quantitative densitometric analyses of all Western blot bands were performed using ImageJ (Image Processing and Analysis in Java software, National Institutes of Health, MD).

Electrophoretic mobility shift assay (EMSA)

Binding activity in intestinal tissue and cell extracts was determined by EMSA. The NF- κB consensus oligonucleotide (Santa Cruz Biotechnology) was end-labeled with [γ -³²P] ATP and T4 polynucleotide kinase. Nuclear protein (10 μg) was incubated for 20 min with gel-shift

binding buffer (10 mmol/L Tris, pH 7.5, 50mmol/L NaCl, 1 mmol/L dithiothreitol, 1 mmol/L EDTA, 5% glycerol, and 1 µg of poly [dI-dC]) and 1 µl of labeled probe. Gel-loading buffer was added to the mixture and samples were electrophoresed on a 5% polyacrylamide gel. Unlabeled probe was used as a competitor.

Histologic evaluation

Mouse intestinal tissues (jejunum and ileum) from control, I/R and 15d-PGJ₂ + I/R groups were fixed and paraffin-embedded for further analysis. Control and I/R sections (5 µm) were prepared for histochemical analysis using hematoxylin-eosin staining method. Sections were evaluated for NEC injury by a pathologist in a blinded fashion as previously described [20].

RESULTS

I/R induces rapid PPAR-γ expression in the small intestine

To determine the PPAR-γ expression levels in the small intestine during I/R-induced injury, we analyzed mouse intestinal tissue lysates from sham and I/R groups by Western blotting. The PPAR-γ expression in both jejunum and ileum was significantly increased at 30 min and 1 h after I/R injury when compared to sham group (Fig. 1); this increase returned to baseline by 3 h time point. In contrast, PPAR-γ expression steadily declined to baseline expression levels by 3 h following a similar peak at 30 min after I/R injury in the ileum. These findings demonstrate a rapid intestinal PPAR-γ activation during NEC with regional induction differences in proximal and distal segments of the small intestine, suggesting that the protective effect of PPAR-γ activation during I/R injury is more sustained in proximal small bowel when compared to distal segments of injured intestine during NEC.

15d-PGJ₂ treatment attenuates I/R injury-induced activation of intestinal NF-κB

Next, we examined the PPAR-γ-mediated induction of NF-κB during I/R injury. To demonstrate the protective effect of PPAR-γ, we analyzed proximal and distal intestinal tissues from animals pretreated with 15d-PGJ₂ ligand during I/R injury using EMSA (Fig. 2). The specificity of binding was confirmed by competition with a molar excess of unlabeled probe. A significant induction of NF-κB binding activity was observed during I/R-induced intestinal injury with attenuated response in 15d-PGJ₂-pretreated jejunum, whereas, the protective effect of PPAR-γ agonist in distal small intestine was less pronounced. The attenuated NF-κB activation demonstrates a differential protective effect of PPAR-γ agonist in proximal small intestine when compared to ileum. These results, taken together with the previous finding of differential PPAR-γ expression during I/R injury, may help explain the common clinical finding of ileal rather than proximal small bowel injury in neonates with NEC.

Effect of 15d-PGJ₂ on the small intestine during I/R injury of NEC

Histologic evaluation of murine intestinal sections from NEC and 15d-PGJ₂-pretreated groups showed significant villous destruction with I/R injury in both jejunum and ileum. These findings were consistent with the histologic findings of NEC (Fig. 3). However, pretreatment with 15d-PGJ₂ resulted in significant architectural preservation of villi in both proximal and distal small intestine during I/R-induced NEC injury. Hence, histologic evidence from 15d-PGJ₂-pretreated intestinal sections supports our hypothesis that PPAR-γ agonist pretreatment is protective for small bowel during I/R-induced NEC.

Effect of 15d-PGJ₂ on TNF-α-induced activation of NF-κB in HT-29 cells

For in vitro studies, we induced TNF-α-mediated inflammatory injury in HT-29 intestinal cells at various time points to examine the effect of 15d-PGJ₂ ligand on NF-κB activation using EMSA (Fig. 4). As expected, TNF-α alone induced increased NF-κB binding activity in HT-29

intestinal cells. Pretreatment with 15d-PGJ₂ resulted in significant attenuation in NF-κB activity with a 5 μm treatment. Continued reduction in NF-κB activation was noted at increasing pretreatment concentrations of 15d-PGJ₂. These findings further support our hypothesis that PPAR-γ activation plays a potential early protective role during I/R-induced injury and cellular inflammatory response in the small bowel. Moreover, our results suggest a regulatory effect of PPAR-γ activation on NF-κB expression in NEC.

DISCUSSION

Ischemia-reperfusion injury of the gastrointestinal tract can manifest in various ways, including pathologic conditions such as septic shock, hemorrhagic shock, and acute mesenteric ischemia [21;22]. Reperfusion injury is induced through various mediators that include ROS, nitric oxide, and effector cells involved in inflammatory cascade activation [14;23;24]; however, the exact role of I/R-induced injury in the pathogenesis of NEC remains unclear. We have previously demonstrated that TNF-α induces significant intestinal epithelial cell apoptosis *in vitro*, and may play an important role in intestinal mucosal injury during NEC (unpublished data). In addition, NF-κB activation has been shown to contribute to gut injury and inflammation associated with NEC by activating various cytokines and inflammatory mediators. Although the exact mechanism of NF-κB mediated gut injury remains unknown, the effect on inflammation of NF-κB inhibition revealed in this study and others strongly supports the central role of this protein in the pathogenesis of gut injury. Consequently, modulation of cellular inflammatory response pathways during the early period of intestinal injury may prove to be beneficial in overall survival rates of neonates with NEC.

In this study, we show a rapid induction of PPAR-γ expression and increased NF-κB binding activity in the small bowel during *in vivo* NEC, suggesting a temporal relationship of PPAR-γ regulation of the NF-κB pathway in murine small bowel as a result of I/R injury. Furthermore, we observe significant attenuation of NF-κB binding activity with 15d-PGJ₂ pretreatment, suggesting an important early protective effect for PPAR-γ during I/R-induced intestinal injury *in vivo*. Histologic changes induced by intestinal I/R injury alone and in combination with 15d-PGJ₂ pretreatment demonstrate significant preservation of intestinal architecture in 15d-PGJ₂-pretreated intestinal tissues in contrast to I/R injury alone. These findings support our hypothesis that PPAR-γ may regulate the early inflammatory response during I/R injury and protect small intestine from significant injury during NEC. Regional differences in sustained intestinal PPAR-γ expression during I/R injury suggest an important differential intestinal response to injury and inflammation despite equally robust early PPAR-γ expression in both proximal and distal bowel. Although, the mechanism involved in this response is unclear, this finding may help to explain why in neonates with NEC the distal small bowel is frequently involved, whereas, jejunal disease is rather rare. Further studies are necessary to address this point. Some investigators have shown that in the absence of another PPAR family member, PPAR-α, ileal permeability is enhanced during experimental colitis and can induce multi-organ failure [25;26]. Similarly, exogenous PPAR-α ligand application can reduce intestinal injury during splanchnic artery occlusion-induced shock [24]. These studies collectively support our *in vivo* and *in vitro* findings that preconditioning of the small intestine with potent PPAR-γ ligand, 15d-PGJ₂, exerts a significant protective effect during I/R injury by modulating cellular inflammatory response, enhancing PPAR-γ expression and preserving architectural integrity of the small intestine that is normally compromised during NEC. In summary, the present work demonstrates that ischemia-reperfusion injury induces activation of PPAR-γ and NF-κB expression in small intestine. In addition, the activation of NF-κB is known to correlate with increased gut injury. Our results from this study illustrate that subsequent intestinal injury can be diminished with early activation of PPAR-γ via treatment with its ligand, 15d-PGJ₂. Induction of PPAR-γ and activation by its endogenous agonist 15d-PGJ₂, as well as direct anti-inflammatory effects of this ligand, contributed to the attenuated NF-κB response observed

after I/R injury to the small intestine. Similarly, our *in vitro* results also demonstrate inhibition of NF- κ B activity in the presence of increased PPAR- γ ligand concentrations, suggesting a central role of PPAR- γ activity in regulating NF- κ B mediated gut injury in NEC. Modulation of this process, specifically the activation of PPAR- γ and consequent inhibition of NF- κ B expression, may represent a beneficial therapeutic approach in neonates with NEC.

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Fig. 1. I/R induces early PPAR-γ expression in the small intestine

Transient occlusion of superior mesenteric artery for 45 min was followed by variable period of reperfusion (0.5, 1, 3 h). Western blot analysis demonstrates early I/R-induced PPAR-γ expression in the small intestine at 30 min and 1 h. This response diminished significantly at 3 h with residual jejunal and ileal PPAR-γ expression. β-actin indicates equal protein loading.

Fig. 2. 15d-PGJ₂ attenuates I/R injury-induced NF-κB activation

I/R injury induces activation of NF-κB in the small intestine, as confirmed by EMSA. In contrast, intestinal sections pretreated with PPAR-γ agonist, 15d-PGJ₂, demonstrate significant attenuation of I/R injury-induced intestinal NF-κB binding activity.

Fig. 3. Effects of 15d-PGJ₂ on small intestinal I/R injury

Representative intestinal sections from mice subjected to I/R injury alone and pretreated with 15d-PGJ₂ are compared with control sections. I/R injury alone induced significant intestinal injury in both jejunum and ileum consistent with NEC (*middle row*). Pretreatment with PPAR- γ agonist, 15d-PGJ₂, significantly attenuated I/R-induced gut injury and preserve structural mucosal integrity similar to the control intestinal sections (*bottom row*).

Fig. 4. Effects of 15d-PGJ₂ on TNF- α -induced activation of NF- κ B in HT-29 cells

HT-29 cells (2×10^4) were pretreated with 15d-PGJ₂ (5–30 μ M; 30 min), and then with TNF- α (1 nM) for 30 min. Nuclear extracts (5 μ g) were analyzed for NF- κ B binding activity using EMSA. Pretreatment with 15d-PGJ₂ attenuated TNF- α -induced NF- κ B binding in HT-29 cells in a dose-dependent fashion.