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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-κB in small intestine. Pretreatment with PPAR-γ agonist, 15d-PGJ_2, attenuated intestinal NF-κB response and I/R-induced gut injury. Activation of PPAR-γ demonstrated a protective effect on small bowel during I/R-induced gut injury.

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 5–10% 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 [2–6]; however, the exact cellular signaling pathways involved in intestinal injury during NEC still remain unclear.

NF-κB is an important transcription factor for the activation of many inflammatory mediators and cytokines [7]. The NF-κB subunits, p50 and p65, are expressed in all cell types and are normally sequestered in the cytoplasm bound to the inhibitory protein IκB. Upon activation, IκB is rapidly phosphorylated and degraded by proteasomes, allowing NF-κB to translocate into the nucleus where it binds to its consensus sequence within the promoter region of various target genes. The activation of NF-κB is involved in several inflammatory conditions, such as inflammatory bowel disease and pancreatitis. Previously, we demonstrated an induction of NF-κB binding activity following I/R injury in the small intestine of rats using an experimental model of NEC [4]. Recently, inhibition of IκB phosphorylation using NBD peptide-affording selective inhibition of NF-κB activity was shown to decrease mortality and bowel injury in an animal NEC model [8]. Therefore, activation/inhibition of upstream regulators of NF-κB 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 three 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 [12–14]. PPAR-γ ligands demonstrate attenuation of the inflammatory response in colon, Alzheimer's disease, arthritis, and aspirin-induced gastric inflammation [15–18]. 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-κB pathway. PPAR-γ has been shown to inhibit NF-κB by associating with the

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p65 subunit in the nucleus and promoting export of the PPAR-γ:p65 complex to the cytosol [7]. However, the role of PPAR-γ in the regulation of NF-κB 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-κB 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, 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 J_2 (15d-PGJ_2), 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; i.p.), 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 intraperitoneal 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.

PPAR-γ ligand, 15d-PGJ_2, 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-PGJ_2 (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 HT-29 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-PGJ_2 (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 (13,200 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-^{32}P] 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, 50 mmol/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-PGJ_2 + 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 the ileum. In contrast, PPAR-γ expression steadily declined to near baseline expression levels by 3 h following a similar peak at 30 min after I/R injury in the jejunum. 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-PGJ_2 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-PGJ_2 ligand during I/R injury

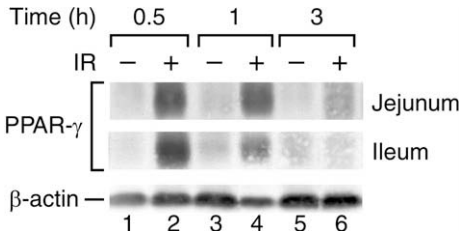

Fig. 1. *I/R induces early PPAR-γ expression in the small intestine.* Transient occlusion of superior mesenteric artery for 45 min was followed by variable period of reperfusion (0.5, 1, 3 h). Western blot analysis demonstrates early I/R-induced PPAR-γ expression in the small intestine at 30 min and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