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ORIGINAL ARTICLE

Endothelin receptor B affects the perfusion of newborn intestine: possible mechanism of necrotizing enterocolitis development

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Abstract

Background Necrotizing enterocolitis (NEC) is one of the most severe gastrointestinal diseases in infancy. Hypoxia is known as one of the major risk factors for the development of NEC. Endothelin, known to regulate vasoconstriction, has two receptors (A and B). However, the role of endothelin receptor B (EDNRB) in neonatal intestinal injury remains unclear. We aimed to investigate whether EDNRB is involved in NEC pathophysiology.

Methods Following ethical approval (#44032), EDNRB hetero knockout mice pups (EDNRB±) and their wild-type (WT) littermates were studied. NEC was induced from postnatal day 5–9 (P5–P9) by hypoxia, gavage feeding of formula and administration of lipopolysaccharide. On P9, the ileum was harvested.

Results NEC induction in WT mice was associated with mucosal injury. However, EDNRB± NEC mice had reduced mucosal injury. Similarly, EDNRB± mice had significantly lower expression of IL-6 mRNA compared to WT NEC mice. Pimonidazole immunostaining was also significantly lower in EDNRB± compared to WT NEC, suggesting reduced tissue hypoxia.

Conclusions Partial knockout of EDNRB results in reduced NEC severity and reduced tissue hypoxia. Intestinal perfusion and hypoxia are important elements of NEC pathogenesis. These findings are relevant to the understanding of NEC pathophysiology and to the development of novel preventive strategies for NEC.

Keywords Necrotizing enterocolitis · Endothelin · Endothelin receptor B · Hypoxia

Introduction

Necrotizing enterocolitis (NEC) is one of the most severe gastrointestinal diseases in preterm infants [1]. Overall survival of infants with NEC has not changed in the past five decades and the mortality is 20–30% [1]. Therefore, it is important to understand detailed etiology of the disease and to establish novel preventive strategy against NEC.

Although NEC is known as a complex and multifactorial disease, some major risk factors have been implicated in its development such as prematurity, bacterial colonization of the gut, and formula feeding. Intestinal mucosa ischemia which is related to prematurity and formula feeding is also one of the important risk factor [2]. Intestinal blood flow is modulated by vasoconstrictor endothelin-1 (ET-1) [3]. ET-1 is a constitutively expressed vasoactive protein produced by endothelial cells that acts as the primary vasoconstrictor in neonatal intestinal vasculature [3, 4]. ET-1 has 2 receptors: endothelin receptor A (EDNRA) and endothelin receptor B (EDNRB). It has been reported that endothelin receptors are expressed at a greater degree in younger age such as suckling period [5]. EDNRA is mainly expressed in vascular smooth muscle cells and induces constriction [3]. EDNRB is expressed in both vascular smooth muscle cells and endothelial cells [3]. Activation of vascular smooth muscle cell EDNRB results in vasoconstriction. Conversely, stimulation

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of endothelial cell EDNRB leads to nitric oxide-mediated vasodilation [3]. It is unclear whether EDNRB contributes to the intestinal injury caused by NEC.

The aim of this study is to investigate whether partial knockout of EDNRB in pup mice influences the severity of experimental NEC.

Methods

Animal

Following ethical approval (#44032), we bred heterozygous EDNRB knock out mice. Heterozygous EDNRB knock out pups (EDNRB±) and their wild type littermates (WT) were used for this study. Prior to the development of experimental NEC, we confirmed partial knockout of EDNRB in the intestine at postnatal day 5 by RT-qPCR. The ratio of EDNRB gene expression in EDNRB± was approximately half (0.442-fold change) compared to WT, suggesting that EDNRB gene expression was properly controlled at the time of NEC induction.

Experimental model of NEC

On P5, pups were randomly assigned into control or NEC groups. Control pups ($n=5$ for each group) were kept with their mother and breastfed without exposure to any stress. Using an established NEC model, NEC pups were separated from their mother from P5 to P9 and NEC was induced ($n=11$ for each group) by giving hypoxia (10 min; 5% O_2, four times a day), gavage administration of lipopolysaccharide (4 mg/kg/day), and gavage of formula feeding (15 g Similac Lower Iron (Abbott Laboratories, Ltd, Saint-Laurent, QC, Canada) + 75 ml Esbilac Puppy Milk Replacer (PetAg, Inc., Hampshire, IL, USA), 152 kcal/100 ml: 50 µl/g of body weight/time, four times a day) [6]. On P9, all mice were intraperitoneally injected with 60 mg/kg pimonidazole (Hypoxyprobe Inc., Burlington, MA, USA), a marker of tissue hypoxia. 90 min after the pimonidazole injection the pup mice were euthanized and the ileum was harvested for staining, RNA and protein extraction.

HE (haematoxylin and eosin) and immunohistochemistry staining

Distal ileum samples were fixed in 4% formalin and embedded in paraffin, then stained with hematoxylin and eosin (HE) or immunohistochemistry for pimonidazole (1:50 anti-pimonidazole antibody from Hypoxyprobe Inc., Burlington, MA, USA). The histology slides were blindly evaluated by three independent investigators (BL, CL and SS) using a published scoring system for NEC severity and pimonidazole levels [7]. NEC was considered present when there was a mucosal injury score of 2 or greater.

RT-qPCR

The expression of messenger RNA (mRNA) in the distal ileum was examined by RT-qPCR. The mRNA was extracted from distal ileum samples by TRIzol Reagent (Thermo Fisher Scientific, Inc., IL, USA). Complimentary DNA (cDNA) was made with qScript cDNA SuperMix (Quantabio, Beverly, MA, USA) and S1000 Thermal Cycler (Bio-Rad Laboratories, Inc). Real-time PCR was performed with Advanced qPCR Master Mix and CFX384 Real-Time System (Bio-Rad Laboratories, Inc). Interleukin 6 (*IL6*) gene expression was analyzed as inflammatory cytokine. *HIF-1α* gene expression was analyzed as the marker for tissue hypoxia. The expression of each gene was normalized to the expression of the housekeeping gene *RPLO*. Sequence of primer for all genes is shown in Table 1.

Protein extraction and Myeloperoxidase (MPO) activity measurement

Ileal tissue was homogenized in tissue extraction buffer (Invitrogen, CA, USA), containing Protease Inhibitor Single-Use Cocktail (Sigma, MO, USA), and protein was isolated. Protein concentration was determined using the Bicinchoninic Acid (BCA) Protein Assay (Thermo Scientific, IL, USA).

Myeloperoxidase (MPO) activity was determined using a Colometric Activity Assay Kit (Sigma Aldrich, St. Louis, MO, USA), and a micro-plate reader with an optical density at 412 nm (Molecular Devices Spectra Max Gemini EM). MPO activity was expressed as U/g protein.

Table 1 List of primers

Gene	Forward sequence (5–3)	Reverse sequence (5–3)
IL6	CCAATTTCCATTGCTCTCCT	ACCACAGTGAGGAATGTCCA
HIF-1α	AAGTGGCAACTGATGAGCAA	GGCGAGAACGAGAAGAAAAA
EDNRB	AAGCCACGCTGTCACTTCTC	GAGGAACGCATCAGACTGGA
RPLO	GGCGACCTGGAAGTCCAACT	CCATCAGCACCACAGCCTTC

Statistics

GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA) was used for statistical analyses. Continuous data were analyzed using *t* test. *P* values < 0.05 were considered significant. Data are quoted as mean and SD.

Results

Intestinal injury and inflammation

Mucosal injury was evaluated by HE staining. NEC induction was associated with higher mucosal injury compared to controls (Fig. 1). However, EDNRB± NEC pups had significantly lower mucosal injury compared to WT NEC.

Ileal inflammation was evaluated by *IL6* RNA expression and MPO activity. Expression of *IL6* RNA and MPO activity was significantly lower in EDNRB± NEC compared to WT NEC (Fig. 2).

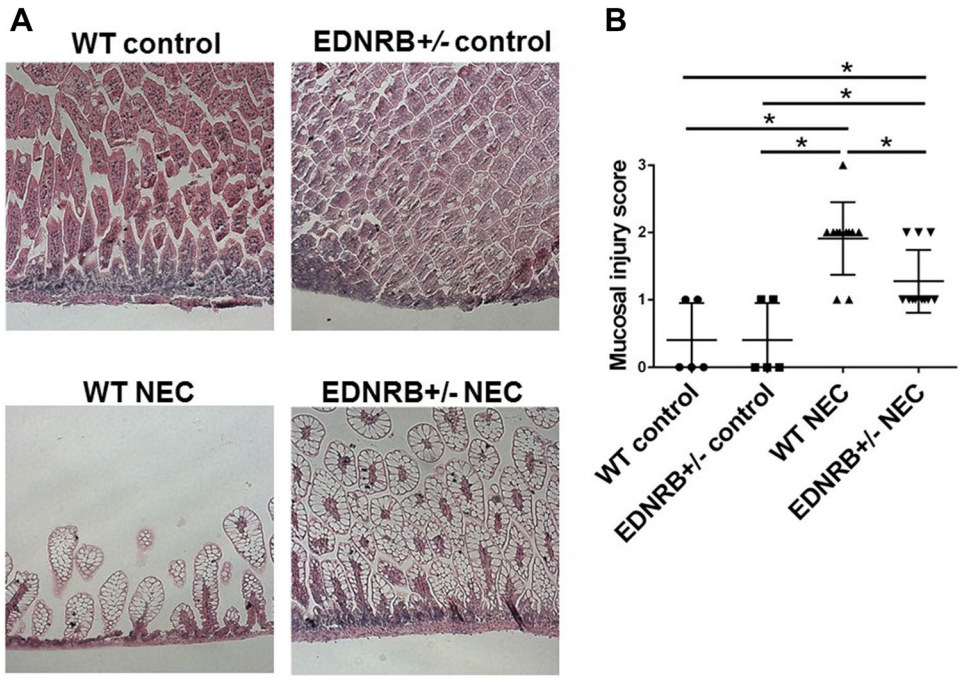

Fig. 1 Partial knockout of EDNRB attenuates ileal mucosal injury. **a** HE staining of control and NEC for both WT and endothelin receptor B hetero knock-out (EDNRB±). **b** Mucosal injury score. NEC induction was associated with higher mucosal injury. However, EDNRB± NEC mice had significantly reduced mucosal injury compared to WT NEC mice. *$p < 0.05$

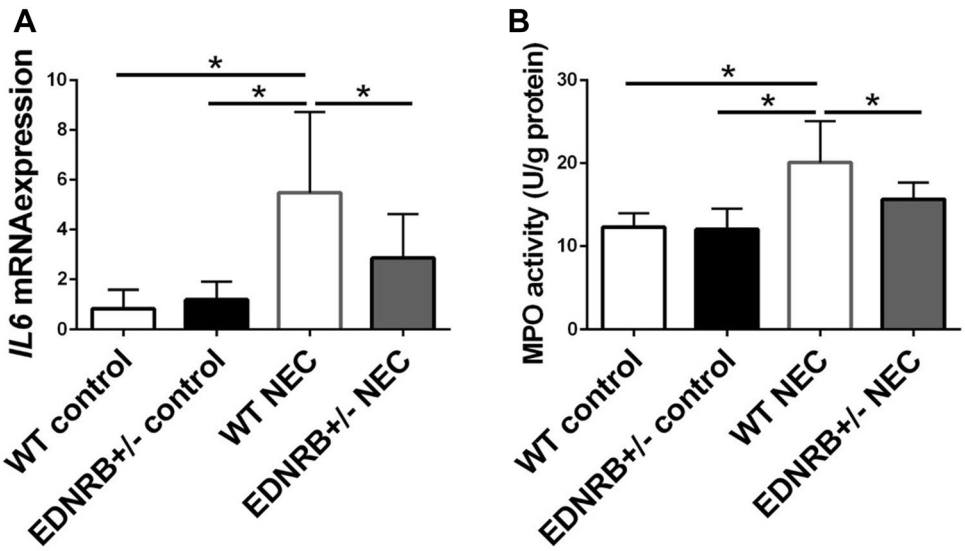

Fig. 2 Partial knockout of EDNRB attenuates ileal mucosal inflammation. **a** qPCR of inflammation markers IL-6 normalized expression in ileal tissue of control and NEC for both WT and endothelin receptor B hetero knock-out (EDNRB±). **b** MPO activity. Inflammation markers of IL6 expression and MPO activity was significantly reduced in EDNRB± NEC mice compared to WT NEC mice. *$p < 0.05$

These results indicate that partial knockout of EDNRB attenuates ileal mucosal injury and inflammation caused by NEC induction.

Intestinal tissue hypoxia

Pimonidazole immunohistochemistry staining was used for evaluation of tissue hypoxia. Pimonidazole accumulated in intestinal epithelial cells in NEC group, but not in control groups (Fig. 3). Average pimonidazole grading was significantly attenuated in EDNRB± NEC compared to WT NEC. Hypoxia inducible factor 1α (HIF-1α) is induced in hypoxic tissue and known to be increased in NEC [8]. Expression of *HIF-1α* mRNA was also evaluated to detect tissue hypoxia. Similar to pimonidazole, *HIF-1α* was significantly lower in EDNRB± NEC compared to WT NEC (Fig. 4). These results indicate that EDNRB± NEC had reduced ischemia in ileal epithelial cells.

Discussion

This study indicates that partial knockout of endothelin receptor B (EDNRB) attenuates intestinal injury and the inflammation caused by NEC induction. Furthermore, EDNRB± pups had less severe intestinal hypoxia compared to WT pups. These results indicate that EDNRB is involved in the development of experimental NEC by attenuating the intestinal ischemia caused by NEC and ultimately reducing disease severity.

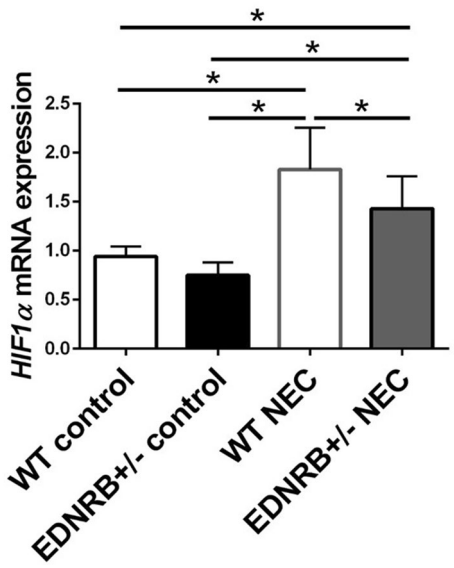

Fig. 4 Partial knockout of EDNRB reduced hypoxia in ileal epithelial cells. qPCR of HIF-1α normalized expression in of control and NEC for both WT and endothelin receptor B hetero knock-out (EDNRB±). Similar to pimonidazole staining, EDNRB± NEC mice had significantly reduced expression of HIF-1α mRNA compared to WT NEC mice $^*p < 0.05$

NEC is known to be a multifactorial disease and several factors are believed to contribute to its pathogenesis [2]. Intestinal ischemia is one important factor in NEC development as supported by the presence of coagulation necrosis in the intestine resected for NEC [2]. In addition, genes related to hypoxia have been reported to be upregulated in human

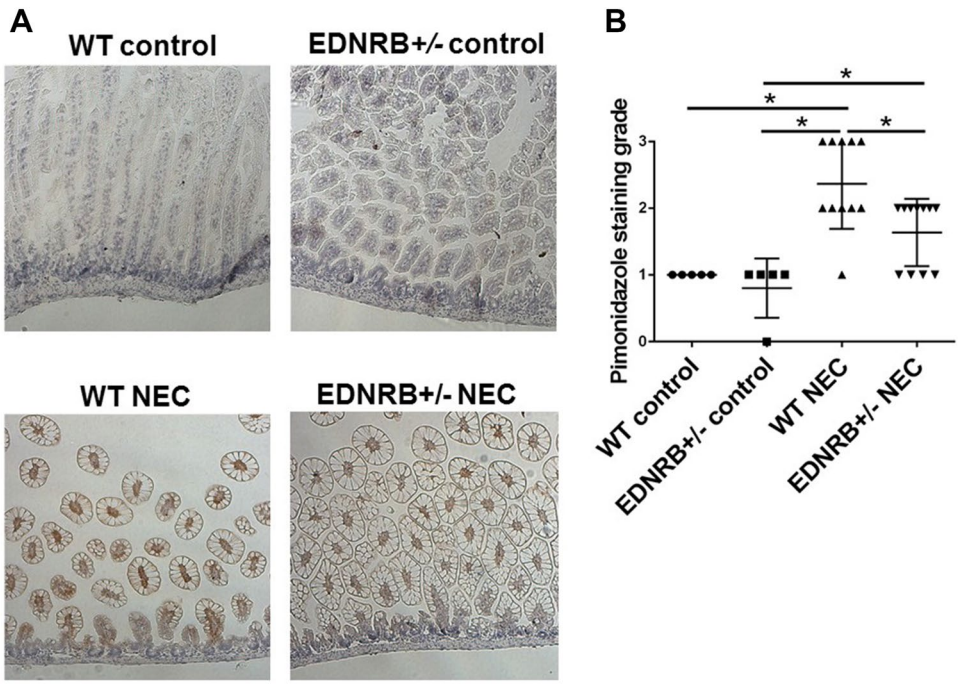

Fig. 3 Partial knockout of EDNRB reduced ischemia in ileal epithelial cells. **a** Pimonidazole immunohistochemistry staining of control and NEC for both WT and endothelin receptor B hetero knock-out (EDNRB±). **b** Pimonidazole staining score. EDNRB± NEC mice had significantly reduced pimonidazole staining score compared to WT NEC mice. $^*p < 0.05$

NEC tissue [8]. We previously reported that reduction of intestinal ischemia resulted in attenuated intestinal injury and inflammation in experimental model of NEC [7]. These results indicate that prevention of tissue ischemia is important to reduce the risk of NEC development.

Endothelin-1 (ET-1) plays an important role in modulating blood flow in the intestine [3]. Ileal ET-1 expression was increased in a rat model of NEC, with compromised microvascular perfusion and decreased intestinal blood flow [9]. Therefore, it is important to understand the role of endothelin receptors in NEC. It is controversial whether EDNRB has beneficial effects on intestinal microcirculation in NEC. Ischemia/reperfusion (IR) injury is considered an important mechanism in NEC pathogenesis [2]. Oktar et al. reported that EDNRB antagonist attenuated mucosal permeability and polymorphonuclear leukocyte infiltration in IR induced mucosal injury model [10]. However, this model is not fully representative of NEC in the first few days of life. Ito et al. reported that EDNRB expression was decreased in rat model of NEC [9]. However, it remains unclear how EDNRB expression is involved in NEC development. In the present study, we directly tested the effects of partial knockout of EDNRB on experimental NEC. We found that EDNRB± NEC mice had lower epithelial hypoxia compared to WT NEC, indicating that partial knockout of EDNRB improved the microcirculation impaired by NEC induction. These results seem to indicate that EDNRB contributes to the prevention of NEC development. Our results further support the involvement of ET-1 related pathway in the pathogenesis of NEC. Inhibition of EDNRB may become new preventive strategy against NEC.

The incidence of NEC varies in relation to the ethnicity of the population studied [11]. Studies evaluating concordance rates in twins have found a familial predisposition for the disease [1]. Although no specific genetic pattern has been clearly associated with NEC, these epidemiological studies suggest the presence of a genetic predisposition to the development of NEC [1, 9]. In addition, it has been reported that EDNRB is one of the top candidate genes to explain the hypoxia adaptation in Ethiopian highlanders who are well-adapted to high altitude hypoxia stress [12]. An experimental study using EDNRB heterozygous knockout mice revealed that partial knockout of EDNRB significantly improved cardiac performance and tissue perfusion under various level of hypoxia [12]. These results indicate that EDNRB plays a key role in hypoxia tolerance. Given the fact that hypoxia is one of the important risk factor for the development of NEC, EDNRB may be an important genetic factor in the development of this disease. Overall, the above studies suggest that genes involved in high-altitude induced hypoxia stress are possible candidate genes for the development of NEC. Further investigations are needed to characterize this genetic issue.

Conclusion

To the best of our knowledge, this is the first study investigating the direct effects of EDNRB on experimental model of NEC. Partial knockout of EDNRB reduced tissue hypoxia and attenuated the disease severity in experimental NEC indicating that EDNRB is involved in the pathogenesis of experimental NEC.

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Compliance with ethical standards

Conflict of interest The author declares that there is no competing interest.

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