Nitric Oxide 66 (2017) 53–61

Contents lists available at ScienceDirect

Nitric Oxide

journal homepage: www.elsevier.com/locate/yniox

The nitric oxide synthase 2 pathway is targeted by both pro- and anti-inflammatory treatments in the immature human intestine

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ARTICLE INFO

Article history:
Received 9 December 2016
Received in revised form
16 February 2017
Accepted 13 March 2017
Available online 14 March 2017

Keywords:
NO synthase 2
Intestinal mucosa
Epithelial cells
Inflammation
Anti-oxidant
Epithelial barrier

ABSTRACT

Background and Aim: NO synthase 2 (*NOS2*) was recently identified as one the most overexpressed genes in intestinal samples of premature infants with necrotizing enterocolitis (NEC). *NOS2* is widely implicated in the processes of epithelial cell injury/apoptosis and host immune defense but its specific role in inflammation of the immature human intestinal mucosa remains unclear. Interestingly, factors that prevent NEC such as epidermal growth factor (EGF) attenuate the inflammatory response in the mid-gestation human small intestine using serum-free organ culture while drugs that are associated with NEC occurrence such as the non-steroidal anti-inflammatory drug, indomethacin (INDO), exert multiple detrimental effects on the immature human intestine. In this study we investigate the potential role of *NOS2* in modulating the gut inflammatory response under protective and stressful conditions by determining the expression profile of NOS2 and its downstream pathways in the immature intestine.

Methods: Gene expression profiles of cultured mid-gestation human intestinal explants were investigated in the absence or presence of a physiological concentration of EGF (50 ng/ml) or 1 μM INDO for 48 h using Illumina whole genome microarrays, Ingenuity Pathway Analysis software and quantitative PCR to investigate the expression of *NOS2* and *NOS2*-pathway related genes.

Results: In the immature intestine, *NOS2* expression was found to be increased by EGF and repressed by INDO. Bioinformatic analysis identified differentially regulated pathways where *NOS2* is known to play an important role including citrulline/arginine metabolism, epithelial cell junctions and oxidative stress. At the individual gene level, we identified many differentially expressed genes of the citrulline/arginine metabolism pathway such as *ARG1, ARG2, GLS, OAT* and *OTC* in response to EGF and INDO. Gene expression of tight junction components such as *CLDN1, CLDN2, CLDN7* and *OCN* and of antioxidant markers such as *DUOX2, GPX2, SOD2* were also found to be differentially modulated by EGF and INDO.

Conclusion: These results suggest that the protective effect of EGF and the deleterious influence of INDO on the immature intestine could be mediated via regulation of *NOS2*. Pathways downstream of *NOS2* involved with these effects include metabolism linked to NO production, epithelial barrier permeability and antioxidant expression. These results suggest that *NOS2* is a likely regulator of the inflammatory response in the immature human gut and may provide a mechanistic basis for the protective effect of EGF and the deleterious effects of INDO.

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http://dx.doi.org/10.1016/j.niox.2017.03.003

Abbreviations

EGF	epidermal growth factor
INDO	indomethacin
IPA	Ingenuity Pathway Analysis
NEC	necrotizing enterocolitis
NO	nitric oxide
NOS2	NO synthase 2

1. Introduction

Necrotizing enterocolitis (NEC) is still a serious gastrointestinal tract disorder of the premature neonate with high morbidity and mortality, despite technological developments and improved care by neonatal intensive care units [1–3]. Although the exact pathophysiology of NEC remains to be elucidated, several lines of evidence suggest that the major contributing factors to the disease include gut mucosal immaturity [4,5], undeveloped intestinal motility [6,7], intestinal ischemia/hypoxia [8,9] and abnormal bacterial colonization [10,11]. Intestinal damage caused by these factors translates into disrupted intestinal epithelial barrier integrity [12], altered inflammatory responses [4], and further increased bacterial translocation, all contributing to the development of NEC.

Gene expression profiles in human intestinal specimens obtained from NEC neonates [13] revealed that nitric oxide synthase (*NOS*) 2 was among the most up-regulated genes suggesting that a dysregulation of this pathway is associated with this intestinal condition. Studies have shown that nitric oxide (NO) could have protective effects in animal models of NEC [14,15]. NO is a short-lived radical product that plays a crucial role in many gastrointestinal physiological and pathological processes, including mucosal blood flow [16], gastrointestinal motility [17,18], epithelial barrier integrity [19], and inflammatory response [20]. NO is produced by the conversion of L-arginine and oxygen into NO and citrulline via constitutive and inducible NOS isoforms. It is mainly the inducible *NOS* isoform *NOS2* that is expressed at high levels under inflammatory conditions [21]. The precise role of NO in gastrointestinal inflammatory disease is still controversial with some studies suggesting that NO is injurious while others are proposing that it is protective [22,23]. These discrepancies could be related to species and model differences [24–27]. Concerning the immature human gut, further work is clearly needed to clarify its specific role and regulation in neonatal intestinal pathologies such as NEC.

Among potential treatments proposed to prevent the onset of NEC or exert healing effects on the intestinal mucosa [2], epidermal growth factor (EGF) has been well-documented for its protective effects [12,28–30]. EGF is a growth factor present in amniotic fluid and maternal milk, which promotes maturation of the developing human gut [31,32]. More recently, we have demonstrated that inflammatory functions were among the main targets of EGF in the human immature intestine [33]. Indeed, using organ culture of the mid-gestation human intestine, transcriptomic analyses revealed that many genes involved in anti-inflammatory and antioxidant responses were up-regulated following exposure to a physiological level of EGF, while expression of pro-inflammatory molecules were attenuated at the same time [33]. In contrast, indomethacin (INDO), a nonsteroidal anti-inflammatory drug, which has been widely used during the neonatal period for the prevention and closure of persistent patent ductus arteriosus in preterm infants [34,35], is associated with increased neonatal complications including a higher risk of developing NEC [36,37]. Treatment of the immature human intestine with INDO in organ culture was found to produce a series of harmful effects on the human intestinal mucosa including the impairment of mitochondrial functions leading to an increase in reactive oxidative species and expression of inflammation-related markers [38].

In the present study, considering the overexpression of *NOS2* noted in the intestines of premature infants affected with NEC, we explored whether *NOS2* could participate in the EGF and INDO-mediated responses in the immature human gut. Gene expression profiles were analyzed in human mid-gestation intestinal explants maintained in organ cultures treated with either EGF (protective) or INDO (injurious) to evaluate the expression of *NOS2* and its downstream pathways.

2. Material and methods

2.1. Tissues

Small intestinal (ileum) tissues were obtained from 15 fetuses ranging from 17 to 20 weeks following legal or therapeutic pregnancy termination with informed patient consent. No tissues were collected from cases associated with known fetal abnormalities or intrauterine fetal demise. Studies were approved by the Institutional Review Committee for the use of human material from the "Centre Hospitalier Universitaire de Sherbrooke/Faculté de Médecine et des Sciences de la Santé".

2.2. Serum-free intestinal organ culture

Ileum samples were prepared as previously described [33,38]. For each tissue, two culture dishes containing approximately 6–9 explants were prepared for each experimental condition. EGF (System Biosciences, Mountain View, CA) was used at a concentration of 50 ng/mL while INDO (Sigma-Aldrich, St-Louis, MO) was added at a final concentration of 1 μM, as previously determined [33,38]. Explants were maintained in culture for 2 days and the medium was changed daily.

2.3. Indirect immunofluorescence

Indirect immunofluorescence was performed on ileal explants as previously described [33]. The primary antibodies used were rabbit anti-NOS2 antibody and anti-claudin 2 (Abcam, Cambridge, MA) used at 1/50 and 1/200, respectively. Secondary antibody was Alexa Fluor 488 anti-rabbit (Invitrogen, Burlington, ON, Canada).

2.4. RNA extraction

RNA was extracted with TRIzol (Invitrogen, Burlington, ON) according to the manufacturer's protocol and stored at −80 °C. For each sample, RNA was quantitated and quality was evaluated by determining RNA integrity values (RIN values > 7.0) as required for microarray experiments.

2.5. Microarray screening and data analysis

Probes for microarray analysis were generated as previously described [33,38]. Briefly, total RNA was isolated from cultured explants for each of the 4 experimental conditions tested (untreated vs INDO-treated; untreated vs EGF-treated) on 4 sets of biological samples obtained from distinct fetuses for a total of 16 samples (4 control ileums and 4 matched INDO-treated ileums, 4 control ileums and 4 matched EGF-treated ileums). All samples were processed, screened (Illumina whole genome Human HT-12 v4 expression bead chips), analyzed and quantile normalized at the microarray platform of the UHN Microarray Centre, University