Pediatric RESEARCH

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BASIC SCIENCE ARTICLE

Impaired antimicrobial response and mucosal protection induced by ibuprofen in the immature human intestine

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BACKGROUND: The use of nonsteroidal anti-inflammatory drugs (NSAIDs) such as indomethacin (INDO) and ibuprofen (IBU) has been shown to be an effective therapy for the closure of patent ductus arteriosus (PDA). However, this treatment has been associated with an increased risk of developing enteropathies in neonates. Whether the use of IBU is safer than INDO for the immature intestine remains to be elucidated.

METHODS: The direct impact of IBU on the human immature intestinal transcriptome was investigated using serum-free organ culture. Differentially expressed genes were analyzed with Ingenuity Pathway Analysis software and compared with those previously reported with INDO. Validation of differentially expressed genes was confirmed by qPCR.

RESULTS: We identified several biological processes that were significantly modulated by IBU at similar levels to what had previously been observed with INDO, while the expression of genes involved in "antimicrobial response" and "mucus production" was significantly decreased exclusively by IBU in the immature intestine.

CONCLUSIONS: Our findings indicate that IBU has a harmful influence on the immature intestine. In addition to exerting many of the INDO observed deleterious effects, IBU alters pathways regulating microbial colonization and intestinal epithelial defense.

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INTRODUCTION

One of the most frequent complications in the clinical management of preterm infants is the persistence of the patent ductus arteriosus (PDA).[1] A persistent PDA in preterm neonates increases the risk of pulmonary congestion and decreased blood flow to vital organs that could lead to various co-morbidities.[2] PDA occurs in about 40–80% of premature infants with low birth weight.[2] The closure of PDA can be achieved effectively with cyclooxygenase (COX) inhibitor administration leading to permanent ductal closure in 60–80% of infants.[3] Nonsteroidal anti-inflammatory drugs (NSAIDs), such as indomethacin (INDO), are the standard pharmacological therapy during the neonatal period for prevention and closure of persistent PDA.[4] However, treatment with INDO is associated with many adverse effects in neonates, mostly affecting the gastrointestinal tract with decreased blood flow and a higher risk of developing necrotizing enterocolitis (NEC) with intestinal perforation.[5,6]

In the last two decades, physicians have used other NSAIDs, namely ibuprofen (IBU), as medical therapy to prevent and treat PDA.[7] Clinical trials have demonstrated that IBU was as effective as INDO in closing the PDA in premature infants.[8–10] Regarding adverse effects, IBU has been associated with milder side effects than INDO on renal and cerebral circulation[9] but a greater risk of triggering severe pulmonary hypertension.[10] While there is still no clear consensus about the best route of administration of IBU in preterm infants with respect to the efficacy and safety profile,[11] treatment with IBU in preterm infants has been associated with gastrointestinal complications, such as spontaneous intestinal perforation,[12,13] suggesting that IBU is not safer than INDO for the premature bowel, which highlights the need of further studies to elucidate those adverse effects.

Recently, we have demonstrated that INDO exerts deleterious effects on the immature human intestine.[14] We also identified that several different metabolic pathways were modulated by INDO such as glycolysis/gluconeogenesis, oxidative phosphorylation, and oxidoreductase activities, resulting in impaired glucose metabolism and mitochondrial function that could lead to a disruption of the intestinal epithelial barrier. In the present study, we combined our model of the mid-gestation human intestine in serum-free organ culture with global gene expression analysis to investigate the direct impact of IBU on the overall physiology of the immature small intestine and compared these effects with those observed previously with INDO. Our results suggest that IBU also exerts deleterious effects on the immature intestinal mucosa, and based on the pathway involved, the sequelae of this treatment could favor bacterial colonization. Consequently, we propose that IBU is not safer than INDO for the immature intestine.

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For the Research Consortium on Child Intestinal Inflammation.

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MATERIALS AND METHODS

Tissues

Small intestinal (ileum) tissues were obtained from 16 fetuses ranging in age 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 of the "Centre Hospitalier Universitaire de Sherbrooke/Faculté de Médecine et des Sciences de la Santé".

Serum-free organ culture

Small intestinal tissues obtained from 16 fetuses were prepared at the same time and under the same conditions as previously described.[14] Briefly, each intestinal tissue was cut into several 5 × 5-mm^2 explants which were maintained in organ culture dishes (Falcon Plastics, Los Angeles, CA) at the interface of a 95% air-5% CO_2 gas mixture and culture medium for up to 2 days (37 °C). For each intestinal tissue, four culture dishes containing approximately 6–9 explants each were prepared, two for each experimental condition (untreated and IBU treated). IBU (Sigma-Aldrich, St-Louis, MO) was added at a concentration of 1 nM to 300 µM for prostaglandin E_2 (PGE_2) production studies, and at 100 µM for gene expression studies, respectively (85% inhibition of intestinal PGE_2 production and in the range of circulating levels in treated preterm babies). Explants were maintained in culture for 2 days and media were changed daily. Studies with INDO were conducted under similar conditions as described previously.[14]

Measure of intestinal PGE_2 levels

The levels of PGE_2 released in the culture medium were measured after 48 h of culture in the presence or absence of various concentrations of IBU (1 nM to 300 µM). Culture media were harvested and the concentration of PGE_2 was determined using a PGE_2 enzyme-immunoassay kit (Correlate-EIA, Assay Designs, Ann Arbor, MI) following the manufacturer's protocol.

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 the quality was evaluated by determining RNA integrity values (RIN values > 7.0) as required for microarray experiments.

Microarray screening and data analysis

Probes for microarray analysis were generated from RNA isolated from two dishes of cultured explants for each experimental condition (untreated vs. IBU treated) tested on four sets of biological samples obtained from distinct fetuses for a total of eight samples (four IBU-treated ileums and four matched control ileums). The biological validity of these experimental conditions was recently established.[15] The eight samples were processed at the microarray platform of the UHN Microarray Centre, University Health Network (Toronto, ON). One Illumina whole-genome Human HT-12 v4 expression beadchip (12 samples per beadchip) was screened, analyzed, and quantile normalized via the UHN Microarray Centre (data are accessible through GEO and are all MIAME compliant). A Wilcoxon test ($p < 0.05$) was used to identify genes expressed differentially (using TMEV 4.2 software). Microarray screening studies for INDO were performed as described previously[14], and the original data are accessible through GEO series accession number GSE38406.

Ingenuity Pathway Analysis

Functional analyses were performed using Ingenuity Pathway Analysis (IPA) 8.8 software (Ingenuity Systems, www.ingenuity.com) as described previously.[14,15] The reference lists containing differentially expressed genes (DEGs) with gene identifiers and corresponding expression values were uploaded into the IPA

Table 1. Primers used in this study

Gene symbol	Sense primer	Antisense primer	Accession no.
AARS	5′-CCAGTGGCAGAAGGATGAAT-3′	5′-GCCATCAGGAGAAAGGTGTT-3′	NM_001605
AGR2	5′-GACAAGCAACAAACCCTTGA-3′	5′-CAAGGTGCCTTCCAGGTAGA-3′	NM_006408
ALDOA	5′-CGTTGTGTGCTGAAGATTGG-3′	5′-AGGCCCTCTGTCTCCTTTTC-3′	NM_000034
APOA1	5′-TGGATGTGCTCAAAGACAGC-3′	5′-CCTTCCCAATCTCCTCCTTC-3′	NM_000039
APOA4	5′-TGAACTCACCCAGCAACTCA-3′	5′-GCAGAAGTCTGAGGGGAGTG-3′	NM_000482
APOB	5′-AGAAAGGCATCTCCACCTCA-3′	5′-GGTTAGCAAGCCAGAAGCTG-3′	NM_000384
CLCA1	5′-GCTCCTGGGGATGATTATGA-3′	5′-TCCTTTGGGATGAGAGCAGT-3′	NM_001285
CYP3A4	5′-CAAGACCCCTTTGTGGAAAA-3′	5′-CGAGGCGACTTTCTTTCATC-3′	NM_017460
DARS	5′-GGAGTCGAAATGGGAGATGA-3′	5′-TTTGGGATTTCTTGGGTCAG-3′	NM_001293312
ENO1	5′-TTGGGAAAGCTGGCTACACT-3′	5′-ATCCCCCACTACCTGGATTC-3′	NM_001428
FABP2	5′-AACTGAACTCAGGGGGACCT-3′	5′-CCTTTTGGCTTCTACTCCTTCA-3′	NM_000134
FCGBP	5′-TCTTCTTCCAGGATGGGATG-3′	5′-GACTAGCAGGGAGCCATCAG-3′	NM_003890
GARS	5′-CTGAGGGACCGTGACTCAAT-3′	5′-ATACCTGGCCTCCACATCAG-3′	NM_002047
HK2	5′-GATTTCACCAAGCGTGGACT-3′	5′-ACAGGTGCTCTCAAGCCCTA-3′	NM_000189
LCN2	5′-TCACCTCCGTCCTGTTTAGG-3′	5′-CGAAGTCAGCTCCTTGGTTC-3′	NM_005564
LDHA	5′-ACTGCAAACTCCAAGCTGGT-3′	5′-CGCTTCCAATAACACGGTTT-3′	NM_001135239
MUC2	5′-CATCACATTCATGCCCAATG-3′	5′-CAGCTCTCGATGTGGGTA-3′	NM_002457
NOS2	5′-CTCTATGTTTGCGGGGATGT-3′	5′-TTCTTCGCCTCGTAAGGAAA-3′	NM_000625
PGK1	5′-CTGTGGGGGTATTTGAATGG-3′	5′-CTTCCAGGAGCTCCAAACTG-3′	NM_000291
PI3	5′-AGCAGCTTCTTGATCGTGGT-3′	5′-ACGGCCTTTGACAGTGTCTT-3′	NM_002638
REG1A	5′-CCAATGCCTATCGCTCCTAC-3′	5′-AATCAGTGAGGCCACAAAGG-3′	NM_002909
RPS3A	5′-CCGGAAGAAGATGATGGAAA-3′	5′-CAAACTTGGGCTTCTTCAGC-3′	NM_001006
TFF3	5′-CTCCAGCTCTGCTGAGGAGT-3′	5′-GAAACACCAAGGCACTCCAG-3′	NM_003226

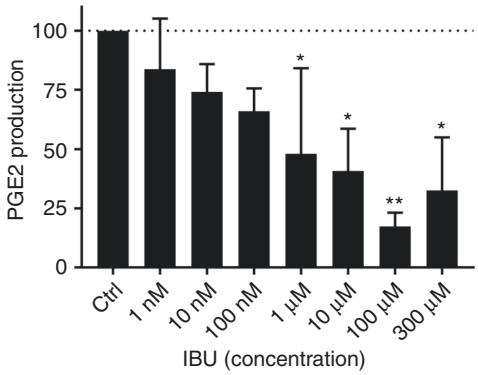

Fig. 1 Inhibition of PGE_2 production by IBU in the human small intestine at mid-gestation. Changes in intestinal PGE_2 levels after 48 h of culture in the presence of increasing concentrations of IBU. Data are expressed as percentage of inhibition of PGE_2 compared to the corresponding untreated control segment. Values shown are the mean of three independent biological samples; $*p < 0.05$ and $**p < 0.001$ vs. untreated control segments

application. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Knowledge Base. IPA allows filtering to consider only functions and interactions in protein networks and/or pathways that are known for the defined species and tissue or cell line range. The stringent filter set for human and relaxed filter for tissues and cell lines were used for the core analyses. Fisher's exact test was used to calculate a *p*-value determining the probability that each biological function assigned to that data set would be due to chance alone.

Data validation by quantitative PCR

RNA from the same 32 samples used for microarray analyses was used for quantitative PCR (qPCR) confirmation. All reactions were performed in duplicate as previously described.[14,15] Briefly, reactions were performed in an Mx3000P real-time PCR system (Stratagene, Cedar Creek, TX) starting with 10 min of Taq activation at 95 °C, followed by 40 cycles of melting (95 °C, 30 s), primer annealing at the temperature appropriate for each primer (55–60 °C, 45 s), and extension (72 °C, 45 s) ending with a melting curve analysis to validate the specificity of the PCR products. Fluorescence data were acquired after each annealing step. Amplification efficiencies ranged from 91% to 105%. Brilliant II SYBR®Green QRT-PCR Master Mix (Stratagene) was mixed with the appropriate primers and high-quality sterile water. The genes investigated were alanyl-tRNA synthetase (AARS), anterior gradient 2 (AGR2), apolipoprotein A1 (APOA1), apolipoprotein A4 (APOA4), apolipoprotein B (APOB), chloride channel accessory 1 (CLCA1), aspartyl-tRNA synthetase (DARS), fatty acid binding protein 2 (FABP2), Fc fragment of IgG binding protein (FCGBP), glycyl-tRNA synthetase (GARS), lipocalin 2 (LCN2), mucin 2 (MUC2), peptidase inhibitor 3 (PI3), regenerating family member 1 alpha (REG1A), and trefoil factor 3 (TFF3). Primers are listed in Table 1 and were generated using the primer formation software Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) with attention given to avoiding primer-dimer formation by stringent use of the maximum 3′ self-complimentarity function of the Primer3 program. Differences in gene expression were evaluated by comparing untreated vs. IBU- or INDO-treated samples for a given intestinal segment using the equation $R = (E_{target})^{\Delta Cttarget} / (E_{reference})^{\Delta Ctreference}$. The reference gene used was the ribosomal protein S3A (RPS3A).

Western blot analysis

Analysis of protein markers in the culture medium was performed as previously described.[16] Briefly, culture media were lyophilized by vacuum concentration (SpeedVac, Savant), resuspended in Laemmli buffer and evaluated for protein content. For each sample, 50 µg of proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis under denaturing conditions and electrotransferred onto nitrocellulose for immunodetection using the primary antibodies anti-LCN2 (ab41105, Abcam, Toronto, ON), anti-REG1A (ab47099, Abcam), and anti-TFF3 (ab108599, Abcam), all diluted 1/1000 in 10% Blotto in PBS and detected using horse radish peroxidase-conjugated secondary antibodies (GE Healthcare-Amersham Bioscience, Baie d'Urfé, QC).

Table 2. Families of pathways modulated by IBU in the immature human intestine

Inflammation-related	Acute phase response signaling
	Arachidonic acid metabolism
	Differential regulation of cytokine production in intestinal epithelial cells by IL-17
	Hepatic fibrosis / hepatic stellate cell activation
	IL-12 signaling and production in macrophages
	IL-17 signaling
	IL-17A signaling in fibroblasts
	LPS/IL-1-mediated inhibition of RXR function
	MIF regulation of innate immunity
	Role of IL-17A in psoriasis
Fatty acid metabolism	Atherosclerosis signaling
	Bile acid biosynthesis
	Fatty acid metabolism
	FXR/RXR activation
	Glycerolipid metabolism
	Linoleic acid metabolism
	LXR/RXR activation
	PXR/RXR activation
Amino acid metabolism	Aminoacyl-tRNA biosynthesis
	Arginine and proline metabolism
	Nitrogen metabolism
	Phenylalanine, tyrosine and tryptophan biosynthesis
	Tryptophan metabolism
	Urea cycle and metabolism of amino groups
Sugar metabolism	Ascorbate and aldarate metabolism
	Fructose and mannose metabolism
	Galactose metabolism
	Glycolysis/gluconeogenesis
	Pentose and glucuronate interconversions
	Pyruvate metabolism
Signaling	Polyamine regulation in colon cancer
	RAN signaling
	TR/RXR activation
	VDR/RXR activation
Oxidoreductase activity	Metabolism of xenobiotics by cytochrome P450
	Production of nitric oxide and reactive oxygen species in macrophages
	Xenobiotic metabolism signaling

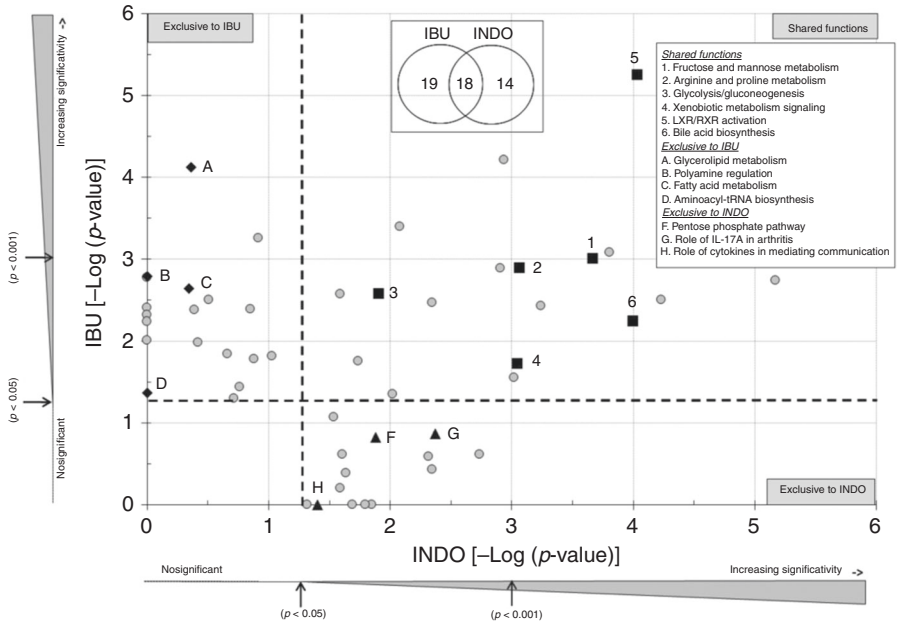

Fig. 2 Cellular, molecular, and physiological functions modulated by IBU and INDO in the human small intestine at mid-gestation. The negative logarithm of *p*-values (Fisher's test), calculated by IPA, for each of the functional categories affected by IBU was plotted against the negative logarithm of *p*-values of the corresponding categories affected by INDO in the small intestine. Insets: Venn diagram showing the 51 molecular and cellular functions identified in IBU and INDO. Of the 37 pathways found in IBU, 18 were also found in INDO. Numbers and letters indicate shared and exclusive functions belonging to IBU and INDO, respectively (listed in the inserted table). Thresholds (dotted lines) denote $p = 0.05$ [$-\text{Log}(0.05) = 1.3$]

RESULTS

Determination of IBU concentration for inhibition of intestinal PGE_2

The dose of IBU used clinically in PDA closure generally aims to use the lowest possible concentration to achieve the desired outcome. Therefore, we first performed a dose–response curve study to determine the appropriate working concentration of IBU to use in organ cultures of mid-term intestinal tissue. Levels of PGE_2 were measured as an indicator of COX activity, the target of NSAID inhibition. As seen in Fig. 1, increasing concentrations of IBU markedly decreased the production and release of PGE_2 by small intestinal explants compared to controls. We observed that IBU significantly inhibited PGE_2 production by almost 85% at concentrations of 100 µM, confirming that intestinal cultures were responsive to IBU. In preterm infants, studies showed that optimum PDA closure rates with IBU could be achieved with plasma levels varying between 90 and 210 µM.[17,18] Based on these data, IBU was used at 100 µM concentration for the studies in organ culture.

Global gene expression analysis of the effect of IBU on the mid-gestation human small intestine

To investigate the modulatory influence of IBU on gene expression profiles in the mid-gestation intestine, matched control and IBU-treated explants prepared from ileums of four different fetuses were cultured in serum-free medium for 48 h. Gene expression profiles were then determined using Illumina wholegenome expression beadchip microarrays providing coverage of more than 47,000 transcripts for each of the eight samples. Statistical analyses revealed that 444 genes were significantly differentially expressed (±1.5 fold change) when IBU was administered in the ileum, in comparison with controls (Supplemental Table S1 for gene list). Of the DEGs, 253 were upregulated and 191 were downregulated. The original data have been deposited into the National Center for Biotechnology Information's Gene Expression Omnibus and are accessible through GEO Series accession number GSE108369.

Pathway analysis of the effect of IBU on the immature human ileum

All significantly DEGs identified in the mid-gestation ileum exposed to IBU were subjected to IPA software analysis which led to the identification of 37 canonical pathways modulated by IBU that can be classified as belonging to six major families of metabolic pathways (Table 2) including inflammation, fatty acid metabolism, amino acid metabolism, sugar metabolism, signaling and oxidoreductase activities. The list of genes associated with each canonical pathway is provided in Supplemental Table S2.

Canonical pathways modulated by IBU and INDO in the mid-gestation human small intestine

To compare the effects of IBU and INDO in the mid-gestation small intestine, all significant DEGs modulated by either IBU or INDO were subjected to IPA comparative analysis of cellular, molecular, physiological and metabolic functions. Plotting the negative logarithm of *p*-values calculated by IPA for each of the functional categories found in IBU - against the negative logarithm of *p*-values of the corresponding categories found in INDO - allowed visualization of the functions that are more relevant to each treatment (Fig. 2), where each point corresponds to one of the 51 pathways. IBU and INDO were found to significantly modulate gene expression of 37 and 32 pathways, respectively. As illustrated with a Venn diagram (Fig. 2, inlet), 18 of these pathways were modulated by both treatments. Supplemental Table S3 lists these functions according to statistical significance in the variation of the expression of genes in each category. Among these common pathways were functions that were previously reported to be associated with NSAID treatment such as "glycolysis/gluconeogenesis", "arginine and proline metabolism", "fructose and mannose metabolism", "LXR/RXR activation", and "bile acid synthesis". Is it noteworthy that numerous pathways were found to be exclusively modulated by either IBU or INDO. For instance, IBU modulated biological processes such as "fatty acid metabolism", "glycerolipid metabolism", "polyamine regulation", and "aminoacyl-tRNA biosynthesis", while pathways such as "role of

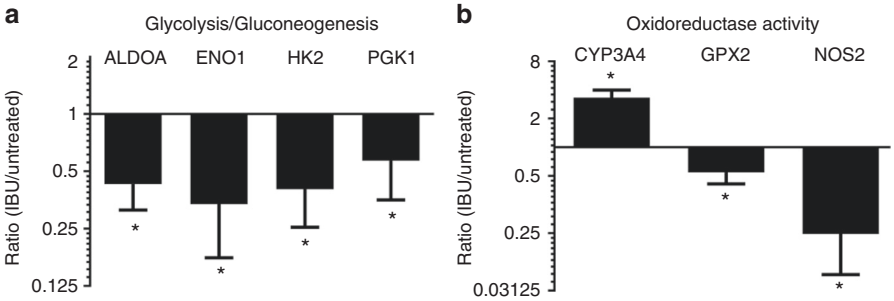

Fig. 3 Differential effects of IBU on markers of glycolysis and oxidoreductase activities in the human small intestine at mid-gestation. Real-time quantitative PCR analysis of transcript levels of **a** glycolytic enzymes (ALDOA, ENO1, HK2, and PGK1), and **b** oxidoreductase activities (CYP3A4, GPX2, NOS2) after 48 h of culture in the presence of IBU (100 μM) in the mid-gestation human ileum. Samples were normalized to RPS3A and data are expressed as ratios of IBU treated over untreated segments, expressed on a Log2 scale. Values shown are the mean of three to four independent biological samples; $*p < 0.05$ vs. corresponding untreated control segments

IL-17A in arthritis", "pentose phosphate pathway", and "role of cytokines in mediating communication between immune cells" were found to be affected exclusively by INDO treatment, suggesting that IBU and INDO exert distinct effects on the immature intestine.

Validation of microarray results by qPCR

As a first step to validate the microarray results, representative genes from two of the common pathways were tested by qPCR. As expected from previous observation with INDO[14,15] IBU treatment induced a significant reduction in the expression of genes of the glycolysis/gluconeogenesis pathway ALDOA, ENO1, HK2 and PGK1, as well as an increase in CYP3A4 and a decrease in GPX2 and NOS2 for oxidoreductase activity (Fig. 3).

We then focused on pathways that appear to be exclusively modulated by IBU using "fatty acid metabolism" and "aminoacyl-tRNA biosynthesis" as archetypes and tested genes representative of these functions by qPCR. As shown in Fig. 4a, we confirmed that apolipoproteins APOA1, APOA4, APOB, and FABP2 were significantly upregulated by IBU in intestinal tissue, while only APOA1 was increased by INDO. Another biological function that was identified by IPA as affected by IBU is "aminoacyl-tRNA biosynthesis". IBU was found to increase the expression of two of the three tested genes (AARS and GARS) while INDO had no effect on their expression (Fig. 4a).

Intestinal-related functions modulated by IBU

We also used some of the other available bioinformatics tools such as David Bioinformatics Resources 6.8 (https://david.ncifcrf.gov/home.jsp) and KEGG GENES Database (http://www.genome.jp/kegg/genes.html) for further mining of the microarray data generated from IBU and INDO treatments and confirmed that the two treatments distinctly affect the immature intestine. Interestingly, this complementary approach led to the identification of two specific intestinal-related functions, "antimicrobial response" and "mucus production", that appear specifically modulated by IBU. Further analyses of specific genes that belong to these pathways were then carried out in both IBU- and INDO-treated explants for comparison. For the antimicrobial response, we observed that three genes involved in this category, namely LCN2, PI3, and REG1A, were significantly downregulated by IBU in the immature Ileum (Fig. 4b), while expression of genes involved in mucus production such as AGR2, CLCA1, FCGBP, MUC2, and TFF3 were all downregulated by IBU. It is noteworthy that none of these genes was found to be modulated by INDO in the immature intestine (Fig. 4b).

We also evaluated the expression of some of these markers at the protein level by measuring their release from the explants into the medium over the last 24 h of culture using Western blot. For these studies, we were able to specifically detect LCN2, REG1A, and TFF3 (Fig. 5a). Using five independent cultures grown under control conditions or in the presence of IBU or INDO, we observed a consistent reduction of immunoreactive LCN2 and REG1A in the media of explants treated with IBU (Fig. 5b, c), while TFF3 levels remained comparable to control conditions (Fig. 5d).

DISCUSSION

IBU and INDO demonstrate equal effectiveness in closing PDA,[8–10] but, the wider use of IBU over INDO appears to come from its apparent milder side effects on renal, cerebral, and mesenteric circulation.[8,9] Furthermore, INDO was associated with a higher risk of developing NEC with intestinal perforation.[5] However, IBU also has been associated with gastrointestinal complications in preterm infants including intestinal perforation,[12,13] emphasizing the need for further research on prenatal intestinal physiology. We showed previously that INDO exerts a deleterious influence on immature small intestinal functions when tested in organ culture.[14] In the present study, we determined the global gene expression profiles of immature human intestinal explants cultured in the presence of IBU in the same serum-free organ culture and compared the direct effects of each NSAID on the human immature intestine.

As expected from our previous study with INDO, analysis of the gene expression profiles revealed that IBU affects a broad range of biological pathways in the mid-gestation human intestine. A large part of these functions was related to inflammation such as "arachidonic acid metabolism", "acute phase response signaling", and "differential regulation of cytokine production in intestinal epithelial cells by IL-17" as well as to various basic metabolisms (sugar, amino acid, glycerolipid, and linoleic metabolisms).

We then proceeded to a direct comparison of the DEGs from small intestinal explants treated with IBU and INDO to generate the pathway analysis. It is noteworthy that as for INDO[14], the IBU concentration used in the organ culture system was based on reported plasma levels in preterm babies treated with IBU[17] and corresponded to an ~85% inhibition of PGE2 production from the mid-gestation small intestine. Interestingly, it was found that about half of the significant functional pathways identified were common to both NSAIDs. Among them were several metabolic clusters involved in glucose metabolism such as "fructose and mannose metabolism" and "glycolysis/gluconeogenesis" including the genes ALDOA, ENO1, HK2, and PGK1, consistent with our previous observations.[14] IBU has been shown to impair gluconeogenesis from lactate and fructose in primary cultured hepatocytes.[19] These results suggest that, like INDO, IBU could alter glucose metabolism and impair mitochondrial function in the intestinal mucosa of preterm infants treated for PDA. Other interesting metabolic pathways shared by the two drugs were involved in oxidoreductase activity, namely "xenobiotic

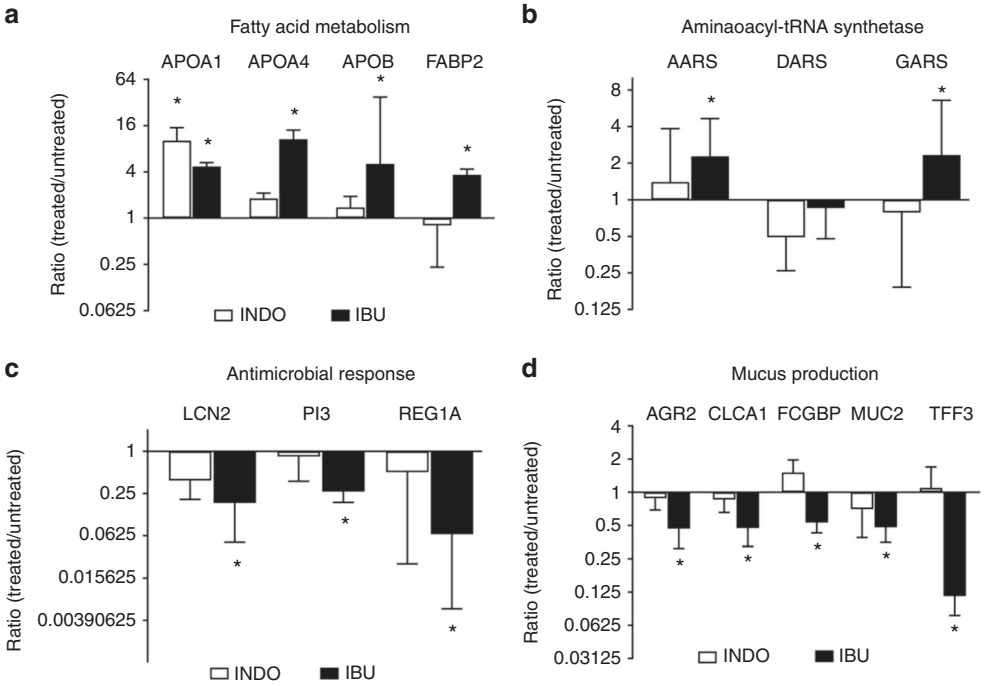

Fig. 4 Differential effects of IBU and INDO on lipid metabolism, aminoacylation, antimicrobial response, and mucus production in the human small intestine. Real-time quantitative PCR analysis of transcript levels of several genes involved in **a** lipid metabolism (APOA1, APOA4, APOB, FABP2), **b** aminoacyl-tRNA synthetases (AARS, DARS, GARS), **c** antimicrobial response (LCN2, PI3, REG1A), and **d** mucus production (AGR2, CLCA1, FCGBP, MUC2, TFF3) after 48 h of culture in the presence of IBU (100 μM) and INDO (1 μM) in the developing human ileum. Samples were normalized to RPS3A and data are expressed as ratios of IBU-treated over untreated segments and INDO-treated over untreated segments. Values shown are the mean of three or four independent biological samples; $*p < 0.05$ vs. corresponding untreated control segments

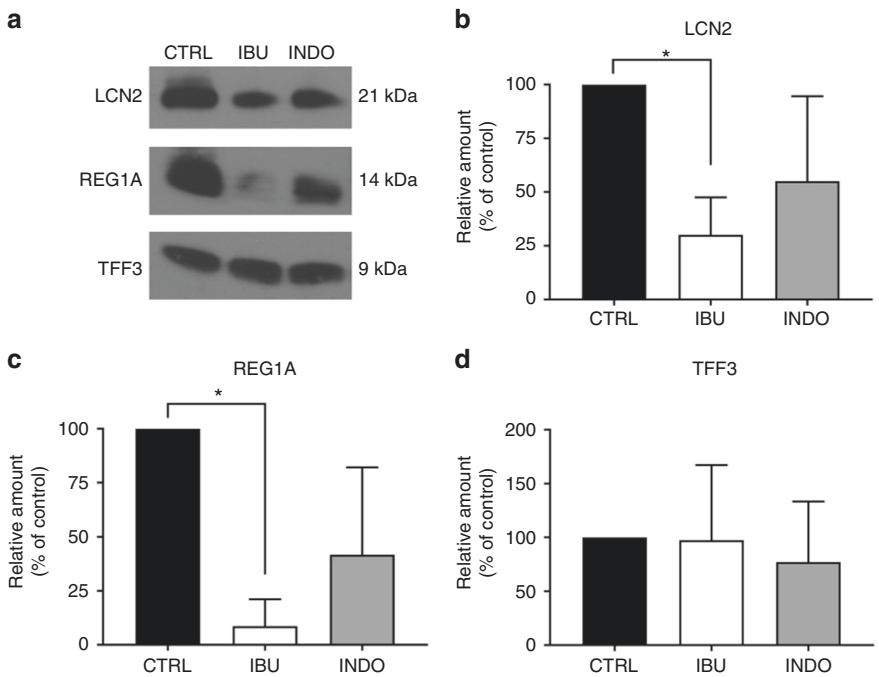

Fig. 5 Differential effects of IBU and INDO on the release of LCN2, REG1A, and TFF3 proteins into the culture medium. **a** Representative Western blot analysis of LCN2, REG1A, and TFF3 immunodetected in 48 h culture media from explants cultured in the presence of 100 μM IBU or 1 μM INDO vs. control conditions (CTRL). Relative amounts of LCN **b**, REG1A **c**, and TFF3 **d** were calculated from independent cultures. Data were expressed relative to CTRL; $*p < 0.05$ vs. corresponding untreated control using one-way Anova test

metabolism signaling" and "production of nitric oxide and reactive oxygen species in macrophages". At the gene expression level, we observed that the immature intestine exposed to IBU induced a significantly increased expression of CYP3A4, one of the most common CYP450 genes in the intestine that is involved in the metabolism of several drugs used in clinics.[20] Moreover, expression of the NOS2 transcript was downregulated in the presence of IBU in the immature ileum. We have recently shown that NOS2 plays a pivotal role in the inflammatory response of the immature intestine being targeted either by anti- or pro-inflammatory effectors.[15]

We next analyzed specific functional pathways that were exclusively modulated by IBU in the immature intestine. As mentioned above, there were a number of significantly modulated metabolisms in this category. Fatty acid metabolism was among these pathways. At the individual gene level, expressions of various apolipoproteins, namely APOA1, APOA4, APOB, as well as FABP2, were found to be specifically upregulated by IBU. The mechanism leading to an upregulation of these apolipoproteins after IBU treatment is not known but may involve the activation of the peroxisome proliferator-activated receptor isoforms, PPARα and PPARγ.[21,22] It is also interesting to note that the binding of lipophilic drugs such as IBU to FABP2 can facilitate its transport into intestinal cells.[23]

Another pathway exclusively modulated by IBU is "amino acid metabolism". We have noted that the expression of two aminoacyl-tRNA synthetases (aaRSs), namely AARS and GARS, were significantly increased in the intestine exposed to IBU. aaRSs are responsible for the aminoacylation reaction which fuses each amino acid to its cognate tRNA, an essential step for accurate protein synthesis.[24] Interestingly, non-canonical functions of aaRSs have been recently highlighted in diverse biological processes such as inflammation, immune response, and angiogenesis.[25] To our knowledge, this is the first report of the effect of any NSAID on the expression of aaRSs, and suggests a potential role for these proteins on the effect of IBU in intestinal physiology.

In an ongoing effort to better understand the adverse effects of IBU in the immature intestinal mucosa, we further screened DEGs modulated by IBU with other bioinformatic tools leading to the identification of other gene clusters of interest for intestinal homeostasis. Among them was the "antimicrobial response". Analysis of representative genes from this group disclosed the negative impact of IBU on the expression of three antimicrobial molecules suggested to play a preventive role on chronic intestinal inflammation such as LCN2 and PI3[26,27] or on intestinal injury such as REG1A.[28] In support of these observations, we also observed herein a significant reduction in LCN2 and REG1A protein released into the culture medium. Taken together, these findings suggest that the downregulation of the expression of genes of the antimicrobial response could be involved in the specific adverse effect of IBU on the intestine of premature infants.

Another group of genes involved in intestinal epithelial cell protection is "mucus production". All analyzed genes of this category, namely AGR2, CLCA1, FCGBP, MUC2, and TFF3, were significantly downregulated by IBU, a phenomenon not observed with INDO. These proteins are produced by goblet cells and are major components of the intestinal mucus.[29] AGR2 has been reported to be essential for mucus production and its deficiency led to a greater susceptibility to colitis in an experimental mouse model.[30] The goblet cell-derived protein CLCA1, despite not having a role in mucus synthesis or structure, has a possible signaling role in the early immune response in colitis.[31] FCGBP is overexpressed in ulcerative colitis[32] while downregulated in the normal colon.[33] Interestingly, it has been reported that FCGBP can link to TFF3 via a disulfide bridge and that this FCGBP-TFF3 heterodimer is an important structural component of intestinal mucus, in addition to interacting with MUC2.[34] TFF3 is a member of the trefoil factor family and is expressed by goblet cells of the small and large intestine.[35] The protective role of TFF3 in inflammatory bowel disease has been demonstrated by the maintenance of epithelial barrier function through inducing expression of tight junction proteins.[36] Mucus secretion and mucus layer formation act as a protective barrier for the intestinal epithelium from luminal threats. This is exemplified by mice lacking the *Muc2* gene developing colitis similar to patients affected with ulcerative colitis.[37] These data suggest a drastic effect of IBU on mucus production and assembly, which may result in an enabling environment that could weaken mucus layer formation and favor abnormal microbial colonization of the immature intestine. The lack of reduction of TFF3 released into the culture medium is surprising considering its significant decrease at the transcript level and may indicate that IBU targets immature goblet cells (actively transcribing but not yet secreting) while the regulated secretion of mature goblet cells is not directly affected by IBU. Further study is, however, needed to test this hypothesis.

In summary, comparison of the gene expression profiles of both INDO and IBU in the immature human intestine confirmed that these NSAIDs target a number of metabolic and biological pathways such as glucose metabolism and xenobiotic metabolism that are detrimental to the immature intestine. More importantly, these data demonstrated that each drug specifically targets a distinct subset of biological functions at least in the organ culture context. While important metabolic pathways exclusively modulated by IBU were identified, such as fatty acid metabolism and protein synthesis, our findings that IBU specifically triggers a significant reduction in the expression of genes involved in the antimicrobial response and mucus production suggest that this deleterious effect of IBU on intestinal primary defenses may at least in part enhance susceptibility to intestinal infection and inflammation in the immature intestine, thus emphasizing the potential risk of IBU treatment in preterm infants in neonatal intensive care unit (NICU). Nevertheless, considering the inherent limitation of an ex vivo approach such as organ culture, further studies are required to investigate this possibility in the more general context of NICUs.

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ADDITIONAL INFORMATION

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REFERENCES

1. Prescott, S. & Keim-Malpass, J. Patent ductus arteriosus in the preterm infant: diagnostic and treatment options. *Adv. Neonatal Care* **17**, 10–18 (2017).
2. Hagadorn, J. I. et al. Trends and variation in management and outcomes of very low-birth-weight infants with patent ductus arteriosus. *Pediatr. Res.* **80**, 785–792 (2016).
3. Mitra, S. et al. Effectiveness and safety of treatments used for the management of patent ductus arteriosus (PDA) in preterm infants: a protocol for a systematic review and network meta-analysis. *BMJ Open* **6**, e011271 (2016).
4. Benitz, W. E. & Bhombal, S. The use of non-steroidal anti-inflammatory drugs for patent ductus arteriosus closure in preterm infants. *Semin. Fetal Neonatal Med.* **22**, 302–307 (2017).

5. Sharma, R. et al. Prenatal or postnatal indomethacin exposure and neonatal gut injury associated with isolated intestinal perforation and necrotizing enterocolitis. *J. Perinatol.* **30**, 786–793 (2010).
6. Fujii, A. M., Brown, E., Mirochnick, M., O'Brien, S. & Kaufman, G. Neonatal necrotizing enterocolitis with intestinal perforation in extremely premature infants receiving early indomethacin treatment for patent ductus arteriosus. *J. Perinatol.* **22**, 535–540 (2002).
7. Ohlsson, A., Walia, R. & Shah, S. S. Ibuprofen for the treatment of patent ductus arteriosus in preterm or low birth weight (or both) infants. *Cochrane Database Syst. Rev.* CD003481, Issue 2. (2015).
8. Loomba, R. & Nijhawan, K. Ibuprofen versus indomethacin for medical closure of the patent arterial duct: a pooled analysis by route of administration. *Cureus* **7**, e274 (2015).
9. Lin, Y. J. et al. Randomized trial to compare renal function and ductal response between indomethacin and ibuprofen treatment in extremely low birth weight infants. *Neonatology* **111**, 195–202 (2017).
10. Amendolia, B., Lynn, M., Bhat V., Ritz, S. B. & Aghai, Z. H. Severe pulmonary hypertension with therapeutic L-lysine ibuprofen in 2 preterm neonates. *Pediatrics* **129**, e1360–e1363 (2012).
11. Erdeve, O. et al. Oral versus intravenous ibuprofen for patent ductus arteriosus closure: a randomised controlled trial in extremely low birthweight infants. *Arch. Dis. Child. Fetal Neonatal Ed.* **97**, F279–F283 (2012).
12. Tatli, M. M. et al. Spontaneous intestinal perforation after oral ibuprofen treatment of patent ductus arteriosus in two very-low-birthweight infants. *Acta Paediatr.* **93**, 999–1001 (2004).
13. Salama, H. et al. A randomized controlled trial on the use of oral ibuprofen to close patent ductus arteriosus in premature infants. *J. Neonatal-Perinatal Med.* **1**, 153–158 (2008).
14. Perron, N. et al. Deleterious effects of indomethacin in the mid-gestation human intestine. *Genomics* **101**, 171–177 (2013).
15. Ferretti, E. et al. The nitric oxide synthase 2 pathway is targeted by both pro- and anti-inflammatory treatments in the immature human intestine. *Nitric Oxide* **66**, 53–61 (2017).
16. Francoeur, C. et al. Degeneration of the pericryptal myofibroblast sheath by proinflammatory cytokines in inflammatory bowel diseases. *Gastroenterology* **136**, 268–277 e3. (2009).
17. Barzilay, B. et al. Pharmacokinetics of oral ibuprofen for patent ductus arteriosus closure in preterm infants. *Arch. Dis. Child. Fetal Neonatal Ed.* **97**, F116–F119 (2012).
18. Gregoire, N., Desfrere, L., Roze, J. C., Kibleur, Y. & Koehne, P. Population pharmacokinetic analysis of Ibuprofen enantiomers in preterm newborn infants. *J. Clin. Pharmacol.* **48**, 1460–1468 (2008).
19. Castell, J. V., Larrauri, A. & Gomez-Lechon, M. J. A study of the relative hepatotoxicity in vitro of the non-steroidal anti-inflammatory drugs ibuprofen, flurbiprofen and butibufen. *Xenobiotica* **18**, 737–745 (1988).
20. Kolars, J. C. et al. CYP3A gene expression in human gut epithelium. *Pharmacogenetics* **4**, 247–259 (1994).
21. Jaradat, M. S. et al. Activation of peroxisome proliferator-activated receptor isoforms and inhibition of prostaglandin H(2) synthases by ibuprofen, naproxen, and indomethacin. *Biochem. Pharmacol.* **62**, 1587–1595 (2001).
22. Colin, S. et al. Activation of intestinal peroxisome proliferator-activated receptor-alpha increases high-density lipoprotein production. *Eur. Heart J.* **34**, 2566–2574 (2013).
23. Velkov, T. et al. The interaction of lipophilic drugs with intestinal fatty acid-binding protein. *J. Biol. Chem.* **280**, 17769–17776 (2005).
24. Yao, P., Poruri, K., Martinis, S. A. & Fox, P. L. Non-catalytic regulation of gene expression by aminoacyl-tRNA synthetases. *Top. Curr. Chem.* **344**, 167–187 (2014).
25. Sissler, M., Gonzalez-Serrano, L. E. & Westhof, E. Recent advances in mitochondrial aminoacyl-tRNA synthetases and disease. *Trends Mol. Med.* **23**, 693–708 (2017).
26. Oikonomou, K. A. et al. Neutrophil gelatinase-associated lipocalin (NGAL) in inflammatory bowel disease: association with pathophysiology of inflammation, established markers, and disease activity. *J. Gastroenterol.* **47**, 519–530 (2012).
27. Zhang, W., Teng, G., Wu, T., Tian, Y. & Wang, H. Expression and clinical significance of elafin in inflammatory bowel disease. *Inflamm. Bowel Dis.* **23**, 2134–2141 (2017).
28. Imaoka, H. et al. Exacerbation of indomethacin-induced small intestinal injuries in Reg I-knockout mice. *Am. J. Physiol. Gastrointest. Liver Physiol.* **299**, G311–G319 (2010).
29. Rodriguez-Pineiro, A. M. et al. Studies of mucus in mouse stomach, small intestine, and colon. II. Gastrointestinal mucus proteome reveals Muc2 and Muc5ac accompanied by a set of core proteins. *Am. J. Physiol. Gastrointest. Liver Physiol.* **305**, G348–G356 (2013).
30. Park, S. W. et al. The protein disulfide isomerase AGR2 is essential for production of intestinal mucus. *Proc. Natl Acad. Sci. USA* **106**, 6950–6955 (2009).
31. Erickson, N. A. et al. Role of goblet cell protein CLCA1 in murine DSS colitis. *J. Inflamm. (Lond.)* **13**, 5 (2016).
32. Kim, M., Lee, S., Yang, S. K., Song, K. & Lee, I. Differential expression in histologically normal crypts of ulcerative colitis suggests primary crypt disorder. *Oncol. Rep.* **16**, 663–670 (2006).
33. Lee, S., Bang, S., Song, K. & Lee, I. Differential expression in normal-adenoma-carcinoma sequence suggests complex molecular carcinogenesis in colon. *Oncol. Rep.* **16**, 747–754 (2006).
34. Albert, T. K. et al. Human intestinal TFF3 forms disulfide-linked heteromers with the mucus-associated FCGBP protein and is released by hydrogen sulfide. *J. Proteome Res.* **9**, 3108–3117 (2010).
35. Kjellev, S. The trefoil factor family - small peptides with multiple functionalities. *Cell. Mol. Life Sci.* **66**, 1350–1369 (2009).
36. Lin, N., Xu, L. F. & Sun, M. The protective effect of trefoil factor 3 on the intestinal tight junction barrier is mediated by toll-like receptor 2 via a PI3K/Akt dependent mechanism. *Biochem. Biophys. Res. Commun.* **440**, 143–149 (2013).
37. Bergstrom, K. S. et al. Muc2 protects against lethal infectious colitis by disassociating pathogenic and commensal bacteria from the colonic mucosa. *PLoS Pathog.* **6**, e1000902 (2010).