British Journal of Nutrition (2012), **108**, 672–681
doi:10.1017/S0007114511006404
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Prematurity does not markedly affect intestinal sensitivity to endotoxins and feeding in pigs

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(Submitted 25 November 2010 – Final revision received 23 August 2011 – Accepted 24 August 2011 – First published online 5 December 2011)

Abstract

Preterm neonates show enhanced sensitivity to nutrient maldigestion and bacteria-mediated gut inflammatory disorders, such as necrotising enterocolitis (NEC). We hypothesised that preterm birth increases the sensitivity of intestinal nutrient absorption to endotoxins and that feeding after birth reduces this response. Hence, we investigated the postnatal development of nutrient digestive and absorptive capacity in the preterm and term pig intestine, and its responsiveness to endotoxins. Pigs were delivered by caesarean section at preterm (*n* 20) or term (*n* 17) gestation, and the small intestine was collected at birth or after 2 d of colostrum feeding, followed by *ex vivo* stimulation with lipopolysaccharide endotoxins and mixed gut contents collected from pigs with NEC. Brush border enzyme activities were reduced in newborn preterm *v.* term pigs (39–45 % reduction, $P<0{\cdot}05$), but normalised after 2 d of feeding. *Ex vivo* leucine and glucose uptake increased with prenatal age. Bacterial stimulation reduced the nutrient uptake similarly at birth and after 2 d in preterm and term pigs (23–41 % reduction, $P<0{\cdot}05$), whereas IL-6 and TNF-α expression was stimulated only at birth. Toll-like receptor-4 expression increased markedly at day 2 for preterm and term pigs (22–33-fold, $P<0{\cdot}05$) but with much lower expression levels in newborn preterm pigs (approximately 95 %, $P<0{\cdot}01$). In conclusion, digestive and absorptive functions mature in the prenatal period, but are similarly affected by postnatal feeding and bacterial exposure in both preterm and term pigs. Nutrient maldigestion may contribute to NEC development, while a prematurity-related hyper-responsiveness to endotoxins could be less important, at least in pigs.

Key words: Intestinal absorption: Bacterial stimulation: Cytokines: Colostrum: Birth

At birth, the intestine of newborn infants is for the first time exposed to colonising bacteria, including potential pathogens. During this critical transition period, the intestinal epithelial barrier and mucosal immune responses are immature, especially in premature infants. This renders the infant susceptible to bacterial translocation and intestinal inflammatory conditions such as necrotising enterocolitis (NEC)[1,2]. NEC is a severe gastrointestinal disease and a major cause of morbidity and mortality in premature infants. The main risk factors for NEC are prematurity, enteral feeding and bacterial colonisation[3]. Although the exact aetiology has not been clearly delineated, the intestinal bacterial flora plays a critical role in NEC pathogenesis[2–8].

Feeding is an important variable in the acquisition of intestinal flora. There is mounting evidence that mother's milk and particularly colostrum is superior to infant formula in stimulating gut maturation, especially in the preterm infant where the digestive and absorptive functions are immature. Colostrum decreases the incidence of NEC, most probably due to decreased pathogenic bacterial colonisation, promotion of a non-pathogenic microbiota, maturation of the intestinal barrier, and possibly by amelioration of the pro-inflammatory response[2,6,9–14]. Intestinal absorptive functions such as uptake of glucose[15] and leucine[16] are susceptible to stimulation with bacterial ligands as well as dietary factors. It remains to be established whether these functions are reduced by preterm birth, leading to maldigestion, bacterial overgrowth and NEC.

The composition of the microbial flora is complex and provides most of the identified innate immune receptor ligands at high concentrations. Thus, innate immune recognition must be tightly regulated within the gastrointestinal tract to balance the need for both mucosal defence and tolerance to colonising bacteria, thereby avoiding inappropriate immune stimulation[17,18]. Intestinal epithelial cells (IEC) provide the initial point of contact with luminal bacteria and respond to signals

Abbreviations: IEC, intestinal epithelial cells; LPS, lipopolysaccharide; NEC, necrotising enterocolitis; qPCR, quantitative real-time PCR; TLR, Toll-like receptors.

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British Journal of Nutrition

https://doi.org/10.1017/S0007114511006404 Published online by Cambridge University Press

British Journal of Nutrition

https://doi.org/10.1017/S0007114511006404 Published online by Cambridge University Press

from the lumen as well as from the basolateral compartment. With the identification of Toll-like receptors (TLR) on IEC, the intestinal epithelium has been identified as an active participant in innate immune reactions, and 'inappropriate immunological responses to bacterial antigens' continues to be a key hypothesis for the enhanced sensitivity of young mammals to gut inflammatory disorders[19,20].

Epithelial dysfunction is speculated to be developmentally regulated, resulting in distinct differences between the immature and mature intestine with respect to interaction with micro-organisms. Thus, the immature intestine may allow inappropriate bacterial stimulation, leading to increased susceptibility to infections and NEC[19,21]. Therefore, postnatal activation of IEC represents an epithelium-specific adaptive process that may be crucial to facilitate postnatal microbial colonisation and establishment of a stable homeostasis on the intestinal surfaces[17,20]. Infant organ culture studies have shown massive increases (80–100 %) in IL-8 mRNA and protein by lipopolysaccharide (LPS) stimulation of fetal intestines (18–21 weeks gestation) compared to intestines from infants and children[19]. Whether a similar large response occurs in the more developed intestine of NEC-sensitive premature newborns (24–34 weeks gestation) remains to be examined. Increased sensitivity to LPS stimulation has also been shown in murine fetal intestinal tissues, but susceptibility to LPS was lost immediately after birth[20]. Most studies on TLR-mediated recognition by IEC have been done using established cell lines[18,22–25] that do not allow us to determine whether birth induces loss of endotoxin response, and whether this differs between preterm and term neonates.

We hypothesised that preterm birth leads to increased immature digestive and absorptive functions, and increased sensitivity to bacterial endotoxins. We speculated that colostrum feeding would reduce an inflammatory response to endotoxins and stimulate intestinal absorptive functions. Studies on the *ex vivo* isolated intestine allowed us to control carefully for factors *in vivo* that would otherwise interact with intestinal functions and endotoxin sensitivity.

Materials and methods

Animal protocol and experimental design

Preterm pigs were delivered by caesarean section from four sows (Large White × Danish Landrace) at 92 % gestation (day 105) and term pigs from two sows at 100 % gestation (day 115). The pigs were immediately transferred to temperature-regulated incubators (AirShields, Inc.) with extra oxygen supply over the first 12 h postnatally. Pigs were killed either immediately (within 3–6 h after birth; preterm: *n* 14, term: *n* 11) or kept for 2 d (preterm: *n* 6, term: *n* 6) based on stratification according to birth weight and sex. Pigs stratified for 2 d of feeding were fitted with umbilical (4F; Portex) and oro-gastric feeding tube catheters (6F; Pharmaplast) and received mother's serum (12 ml/kg intra-arterially during the first 24 h) for immunological protection[26]. The pigs were nourished every 3 h, first with five boluses of sow's colostrum, subsequent with bovine colostrum (both at 15 ml/kg per 3 h) for 2 d until euthanasia. In each pig, anaesthesia was induced and maintained with isoflurane while collecting organs, followed by euthanasia. Pieces of the middle small intestine (20 cm) were transferred to ice-cold Dulbecco's PBS for immediate *ex vivo* stimulation, and distal small-intestinal tissues were snap-frozen for later analysis of enzyme activity. The animal protocol detailed here followed our standard procedures described in detail previously[6,8]. All procedures were approved by the National Committee on Animal Experimentation, Denmark.

Tissue enzyme activity

Activity of mucosal disaccharidases (maltase, sucrase and lactase) and peptidases (aminopeptidase-N, aminopeptidase-A and dipeptidyl peptidase-IV) was determined in homogenates (homogenised in 1 % Triton X-100) of small-intestinal tissue (proximal, middle and distal sections) using specific substrates as described previously[6]. The amount (μmol) of substrate hydrolysed per min at 37°C was considered to represent one unit of enzyme activity.

Ex vivo tissue stimulation and nutrient uptake

Ex vivo D-glucose and L-leucine uptake in intact tissues from segments of the middle small intestine was measured as described earlier[27] after stimulation of tissues with LPS or luminal contents collected from pigs previously diagnosed with NEC (NEC-microbiota). The luminal content of the total small intestine was collected from a series of previous pigs diagnosed with NEC. The contents were frozen immediately after collection (−20°C) and the samples were thawed and pooled before use (*n* 8, referred to as NEC-microbiota). For the intact tissue stimulations, segments of the small intestine were everted, cut into pieces and mounted as 1-cm sleeves on steel rods (with diameters that approximate those of the intestinal segments (4–5 mm)) with silk ligatures, while kept in ice-cold aerated Ringers solution. The sleeves were pre-incubated for 12 min in 37°C aerated Ringers solution with either LPS (100 ng/ml, from *Escherichia coli* O111:B4; Sigma-Aldrich) or NEC-microbiota (8 × diluted), followed by incubation in an uptake solution for 2 min (37°C aerated Ringers solution with [U-^{14}C]D-glucose (0·4 μmol/l; Perkin Elmer Life and Analytical Sciences) and [^{3}H]leucine (5 nmol/l; Perkin Elmer)). Finally, the sleeves were washed in ice-cold Ringers solution and solubilised in 0·5 ml Solvable (Perkin Elmer). Samples were counted with 3 ml Ultima Gold (Perkin Elmer) in a Tricarb 2100TR Liquid Scintillation Analyzer (Packard Instruments). The use of trace levels of D-glucose provides a sensitive indicator of the activity of the high-affinity, Na-dependent glucose co-transporter 1. Similarly, when using trace levels of leucine, the rates of carrier-mediated transport contribute 80–90 % of the transport in preterm and term pigs at birth[28,29]. In addition to the nutrient uptake measurements, segments of everted tissues were co-incubated in the pre-incubation solution, and the tissues were then snap-frozen in liquid N_2 and stored at −80°C for later analysis of cytokine mRNA

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expression by quantitative real-time PCR (qPCR) analysis. All preparations were performed in triplicates.

Ex vivo *primary cell stimulation and nutrient uptake*

Primary differentiated IEC isolated from sections of the proximal small intestine were stimulated *ex vivo* with LPS for measurement of leucine and glucose uptake. Primary cells were isolated and cultured by modification of Lotz *et al.*[18]. In short, tissues were incubated with 0·05 % dithiothreitol in Dulbecco's PBS at 37°C for 10 min, filled with HyQtase (Thermo Scientific HyClone) and incubated for a further 30 min at 37°C. Single cells were collected into ice-cold Dulbecco's PBS and washed before seeding on Transwell membranes (Corning) with or without 100 ng/ml LPS in Dulbecco's modified Eagle's medium supplemented with 0.06 mg/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml bovine insulin, 10 % fetal calf serum and 10 mM-HEPES pH 7·3 (all from Sigma-Aldrich). The cells were pre-incubated for 3 h at 5 % CO_2 –95 % air at 37°C, followed by 2 min-incubation with uptake solution of [U-^{14}C]D-glucose (0·4 μmol/l; Perkin Elmer), [^{3}H]leucine (5 nmol/l; Perkin Elmer) and 25 mM-D-mannitol in glucose-free Hank's balanced salt solution. Cells were washed in ice-cold 25 mM-D-mannitol in glucose-free Hank's balanced salt solution, lysed with 0·1 M-NaOH and counted in by liquid scintillation counting as described previously.

Quantitative real-time PCR analysis

qPCR was used to measure the expression levels of the pro-inflammatory cytokines IL-6 and TNF-α in segments from the middle small intestine after *ex vivo* stimulation with LPS or NEC-microbiota. TLR-4 expression levels were measured in unstimulated intestinal tissues. Total RNA was extracted from frozen tissue samples (AllPrep DNA/RNA Mini Kit with TRIZOL reagent; Qiagen). The RNA quality (intact rRNA 28s/18s) was evaluated by agarose gel electrophoresis. TLR-4 expression analysis was carried out using a different qPCR detection system than for IL-6 and TNF-α; therefore primer design and qPCR cycles differ from that of IL-6 and TNF-α. The QuantiTect Reverse Transcription Kit (Qiagen) was used for complementary DNA synthesis of IL-6 and TNF-α, and the SuperScript III First-Strand Synthesis system (Invitrogen) for TLR-4. Primers and probes were designed based on public databases (GenBank, NCBI and TIGR, Institute for Genome Research) using the PrimerExpress software version 2.0 (Applied Biosystems) and used for complementary DNA amplification (Table 1). For IL-6 and TNF-α, TaqMan 2 × Fast Universal PCR Master Mix (Applied Biosystems) was mixed with 30 ng of template complementary DNA, and gene-specific primers and probes. The qPCR analysis was performed on the 7900HT Fast Real-Time PCR System (Applied Biosystems) with 20 s at 95°C followed by forty cycles of denaturation for 1 s at 95°C and annealing/elongation for 20 s at 60°C. TLR-4 was analysed by Bio-Rad Real-Time PCR detection system (Bio-radicycler version 3.0a). Amplification was conducted with denaturation for 15 min at 95°C, followed by forty cycles of denaturation for 5 s at 95°C and annealing/elongation for 30 s at 60°C, and a final melting curve analysis. All target genes were normalised to the endogenous reference gene β-actin based on geNorm analysis of several reference genes[30], and the mRNA expression in stimulated tissues is presented as fold change compared to unstimulated control tissues.

Statistical analysis

Data were analysed using SAS (version 8.2; SAS Institute) and values in figures and text are presented as means with their standard errors. Enzyme activities, rates of intact tissue leucine and glucose uptake, and ratios of expression of TNF-α, IL-6 and TLR-4 were analysed by the PROC MIXED procedure with treatment (for stimulated tissues), gestational age and age after birth (except for TLR-4 which was only measured at birth), including the related interaction effects as fixed variables, and pig and litter as random variables. Uptake and expression data were log-transformed before statistical analysis for equal distribution of residuals, and all data analyses

Table 1. Primer and probe sequences used for quantitative real-time PCR

Gene	Accession no.		Sequence 5′–3′	TM (°C)	PCR efficiency (%)
IL-6	NM214399	F	TGCGCAGCCTTGAGGATT	59	107
		R	CCCCAGCTACATTATCCGAATG	59	
		P*	TGCAGTTCAGCCTGAG	69	
TNF-α	NM214022	F	CTTGGGTTTGGATTCCTGGAT	59	110
		R	CTTCCCTGGCAGCCACAT	58	
		P	ACCTGGGACATCTGG	70	
TLR-4	AB188301	F	AAGGTTATTGTCGTGGTGT	47	
		R	CTGCTGAGAAGGCGATAC	50	
β-Actin	DQ845171	F	GGCGCCCAGCACGAT	59	94
		R	GCCGATCCACACGGAGTACT	59	
		P	TCATCGCGCCTCCAG	69	
	AY550069†	F	CCACGAAACTACCTTCAACTCC	55	
		R	GTGATCTCCTTCTGCATCCTGT	55	

TM, melting temperature; F, forward primer; R, reverse primer; P, probe; *TLR*, Toll-like receptor.
* All probes were labelled with FAM (6-carboxyfluorescein) and MGB at the 5′and 3′ end, respectively (dihydrocyclopyrroloindole tripeptide). Primers and probes were purchased at Applied Biosystems.
† Separate *β-actin* gene sequence used for TLR-4 normalisation.

were performed on absolute values. For nutrient uptake and cytokine expression data, results are expressed relative to control. For all comparisons, $P<0{\cdot}05$ was accepted as the critical level of significance.

Results

Intestinal weight and digestive enzyme activities

The relative wet weight (to body weight) of the small intestine and stomach, both increased significantly from birth to 2 d of age, in both preterm pigs (24·5–36·0 and 4·7–7·9 g/kg, both $P<0{\cdot}05$) and term pigs (24·2–47·0 g/kg and 5·2–7·4, both $P<0{\cdot}05$). At 2 d, the intestinal weight was significantly higher in term pigs relative to preterm pigs (23 %, $P<0{\cdot}05$).

At birth, the tissue-specific activities of sucrase, lactase and aminopeptidase-A were reduced in preterm *v.* term pigs ($P<0{\cdot}05$) (Fig. 1). After 2 d of feeding, this difference in enzyme activity for preterm *v.* term pigs was less pronounced, and for maltase the enzyme activity increased in preterm pigs compared to term pigs ($P<0{\cdot}05$). All enzyme activities increased markedly from birth to day 2 (22–75 %, $P<0{\cdot}05$) in both preterm and term pigs, except that the increase in lactase and dipeptidyl peptidase-IV activities was significant only in term pigs. Significant interaction effects between gestational age and age after birth were seen for aminopeptidase-A and sucrase only ($P<0{\cdot}01$).

Nutrient uptake in ex vivo stimulated tissues and primary cells

Basal leucine uptake at birth was lower in pigs born preterm, relative to term (52 % reduction, $P<0{\cdot}05$) (Fig. 2). After 2 d of feeding, the difference between preterm and term pigs was less pronounced and not significantly different. The same trend was seen for glucose uptake, although the relative difference was smaller and here only significant after 2 d of feeding (37 %, $P<0{\cdot}05$). In preterm pigs, the rate of leucine and glucose uptake decreased with 48 and 30 %, respectively ($P<0{\cdot}05$). In term pigs, only leucine was significantly reduced from birth to 2 d of age (52 %, $P<0{\cdot}05$). Considering growth in total weight of the small intestine in response to feeding (31 and 48 % for preterm and term pigs, respectively), the total nutrient absorptive capacity of the intestine increased within the 2 d of feeding only in term pigs (17 and 53 % for leucine and glucose, respectively, $P<0{\cdot}05$). No interaction effect between gestational age and age after birth was observed for leucine and glucose ($P=0{\cdot}25$ and 0·37, respectively).

Bacterial stimulation of intestinal tissues reduced the uptake capacity of leucine and glucose (Fig. 3). The strongest effect on uptake was observed when stimulating with NEC-microbiota, which reduced the absorption of leucine at birth and after 2 d of feeding in both preterm and term pigs (32–39 % reductions, all $P<0{\cdot}05$) and similar effects were observed for glucose (23–41 % reductions, all $P<0{\cdot}05$). Stimulation with LPS showed the same trend as for the NEC-microbiota, but the

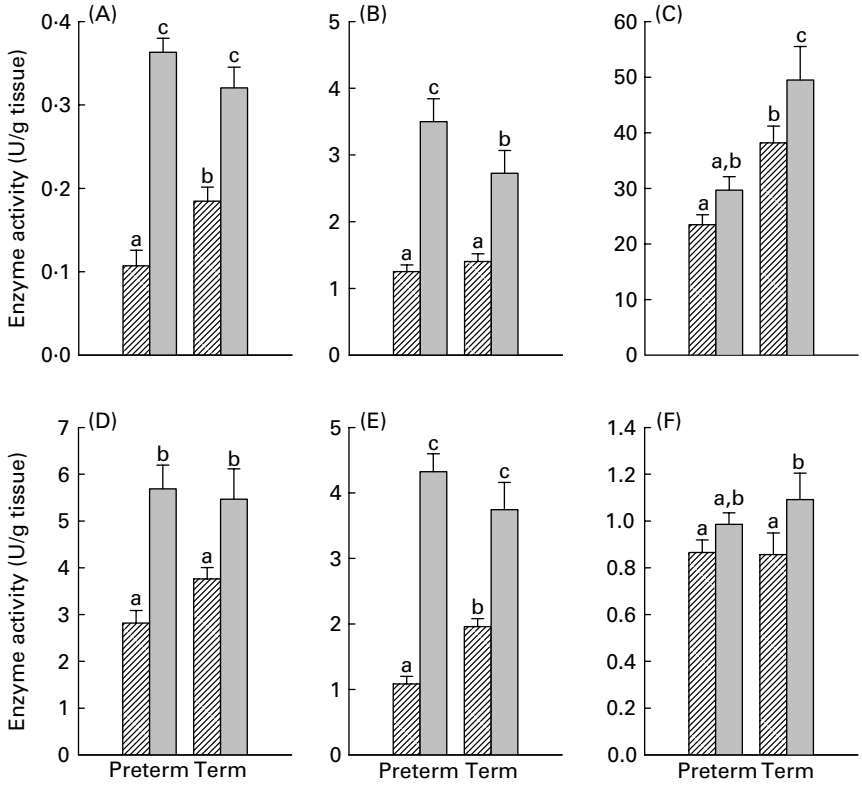

Fig. 1. Brush border enzyme activities in the middle small intestine. (A) Sucrase, (B) maltase, (C) lactase, (D) aminopeptidase N, (E) aminopeptidase A and (F) dipeptidyl peptidase-IV. The amount (μmol) of substrate hydrolysed per min at 37°C was considered to represent one unit of enzyme activity. ▨, Birth; ▩, day 2. Values are means with their standard errors represented by vertical bars (n 9–14). ^{a,b,c} Mean values with unlike letters were significantly different between groups ($P<0{\cdot}05$).

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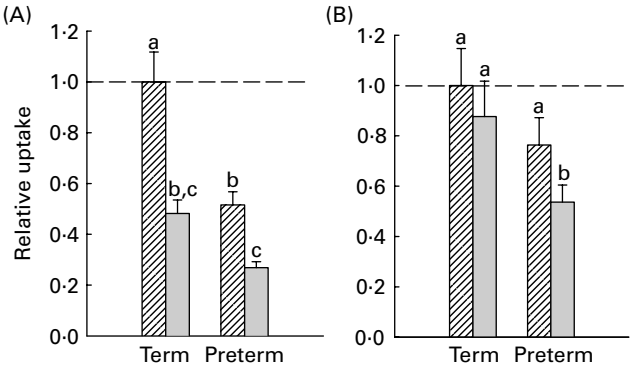

Fig. 2. Relative *ex vivo* uptake of (A) leucine and (B) glucose in unstimulated tissues at birth (▨) or day 2 (▩) in term and preterm pigs, respectively. Tissues were collected from the middle small intestine. Data are shown as fold change relative to term pigs at birth and values are means with their standard errors (n 6–14). [a,b,c] Mean values with unlike letters were significantly different between groups ($P<0·05$).

effects of LPS were lower and only significantly reduced the uptake after 2 d of feeding. There was no difference in response to LPS and NEC-microbiota stimulation between age at delivery (preterm *v.* term) or age after birth (newborn *v.* 2-d-old fed animal). Statistical analysis showed interaction between gestational age and age after birth for both leucine and glucose ($P<0·001$), but no significant interaction with treatment.

Increased bacterial exposure (3 h) in primary IEC resulted in disparity between term and preterm birth with regard to nutrient uptakes. There was no effect of stimulation of cells collected from term pig intestines, whereas in preterm pigs stimulation with NEC-microbiota significantly decreased leucine and glucose uptake after 2 d of colostrum feeding (52 and 54 %, respectively, both $P<0·05$).

Expression of inflammatory cytokines and Toll-like receptors

The tissue expression of the pro-inflammatory cytokines, TNF-α and IL-6, showed relatively modest responses in both preterm and term pigs to stimulation with LPS or the NEC-microbiota (Fig. 4). Hence, the expression of TNF-α increased significantly in LPS-stimulated tissues from newborn term pigs (4·3-fold, $P<0·05$), while IL-6 mRNA was significantly increased following stimulation with NEC-microbiota in newborn preterm pigs (4·6-fold, $P<0·05$) (Fig. 4). No significant interaction was observed between treatment, gestational age and age at birth. The generally higher bacterial responsiveness at birth, relative to 2 d, was not associated with a correspondingly higher TLR-4 expression at birth (Fig. 5). In contrast, TLR-4 expression increased markedly from birth to day 2 in both term pigs (33-fold increase, $P<0·005$) and preterm pigs (22-fold increase, $P<0·05$). At both time points, preterm pigs had much lower intestinal TLR-4 expression level than term pigs (approximately 95 % relative to term pigs, $P<0·01$).

Discussion

The signalling pathways leading from LPS to TLR-4 and NF-κβ activation in IEC have been described in detail[22–25,31], but it remains poorly understood if gestational age at birth has important effects on endotoxin responses just after birth[32] and how this relates to intestinal functions, such as nutrient uptake[15,16,33,34]. We investigated this by *ex vivo* bacterial endotoxin stimulation of intestinal tissues from newborn or 2-d-old pigs delivered by caesarean section preterm at 92 % gestation or at full term. At the chosen stage of prematurity, pigs show many of the clinical signs of prematurity as infants born at 28–32 weeks gestation, a stage of maturity when both pigs and infants show markedly elevated sensitivity to NEC lesions[35]. At this stage of maturity, bacterial endotoxin stimulation reduced nutrient absorption and increased the expression of some pro-inflammatory cytokines, but to a similar extent in preterm and term pigs. In this case, 2 d of colostrum feeding dampened the immune response while the nutrient uptake capacity per g tissue was unchanged. Factors others than elevated immaturity-related endotoxin sensitivity appear to explain that such infants and pigs are more susceptible to NEC. Factors such as immature gastrointestinal motility, leading to intestinal stasis and bacterial overgrowth, together with factors such as intestinal ischaemia, hypoxia and reduced mucosal barrier function *in vivo*, may be more important.

Our data demonstrate that the basal intestinal uptake capacity for leucine and glucose per mg of tissue increases towards normal term, consistent with earlier studies[29]. The uptake decreases within 2 d of feeding in both preterm and term pigs, indicating that the intestine has the highest relative expression of nutrient transporters at birth, reflecting the need to have a high nutrient absorptive capacity just after birth. Alternatively, it is possible that the postnatal decrease reflects that the underlying tissue grows faster than the expression of nutrient transporters, as stimulated by oral feeding and hormones such as glucagon-like peptide-2 and insulin-like growth factor-1[36]. However, as both intestinal length and weight increase rapidly with postnatal age and oral feeding (relative to fasting or intravenous nutrition), the total absorptive capacity may remain the same in fed newborns, or even increase, despite that nutrient uptake per g tissue decreases. The activity of several brush border enzymes corresponded with the leucine and glucose absorption data with regards to lower activity of sucrase, lactase and aminopeptidase-A at birth in preterm *v.* term newborn pigs. After 2 d of feeding, the differences between preterm and term pigs were less pronounced and all enzyme activities increased, suggesting that enzymes in the brush border are regulated faster and correlate better with tissue growth than nutrient uptake transporters. For a few of the enzymes, no difference in activity was seen between the premature and mature tissues, both at birth and after 2 d. This would indicate that the premature tissue is otherwise as viable and functional as the term tissue, and further comparisons between the two for stimulation studies are possible.

For a more realistic array of bacteria and endotoxins related to NEC *in vivo*, we chose to stimulate the intestinal tissues not only with the Gram-negative bacterial ligand LPS, but also with a crude mix of intestinal contents collected from the small intestine of pigs with NEC. The contents typically

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https://doi.org/10.1017/S0007114511006404 Published online by Cambridge University Press

present in our NEC pigs are described in detail in the recent study by Cilieborg *et al.*[7].

As we have shown in earlier studies[7,8], NEC outbreak is probably not closely associated with a specific microflora of the gut contents. Rather, the bacterial components of the microbiota could be more important.

Stimulation of tissues with either LPS or NEC-microbiota generally decreased the mean absorption of leucine and glucose, but we saw no difference between the intestines from preterm and term pigs. Hence, nutrient transporters are sensitive to endotoxin stimulation, but prematurity at birth is not an important additional factor. Earlier studies in adult rabbits have shown a similar ratio of reduction in leucine uptake for the same concentration of LPS[16]. Together, these results indicate that leucine transport is not particularly affected by prenatal age. In children with cancer, overall leucine absorption was not compromised during chemotherapy-induced mucositis, indicating that intestinal inflammation does not markedly affect intestinal leucine absorption. To what extent the specific activity of the transporters at locally infected areas is affected remains unanswered[37]. Interestingly, this would suggest that the observed reduction in local active leucine absorption may be of less importance for the overall intestinal absorptive capacity. Fructose absorption has also been shown to be affected by LPS stimulation of tissues in rabbits[34,38]. Although fructose uses the GLUT-5 transporter for uptake in the apical membrane, and glucose and galactose are transported by Na-dependent glucose co-transporter-1, there is a consistent trend for monosaccharide transport to be affected by endotoxin stimulation. Studies on very immature intestines of human subjects or rodents indicate that the intestine acquires immunological tolerance to endotoxin after birth after a hyper-responsive state in the fetus[19,20]. However, a degree of prematurity similar to that in NEC-sensitive preterm infants, did not increase the intestinal endotoxin responses in pigs. The uptake of leucine and glucose was not less affected by endotoxin with advancing gestational age or time after birth; hence, these important absorptive functions did not acquire an apparent endotoxin tolerance. In relation to the clinical setting, it is important to note that this study only investigates the influence of NEC-related bacteria and endotoxins on the intestinal nutrient uptake capacity. It may

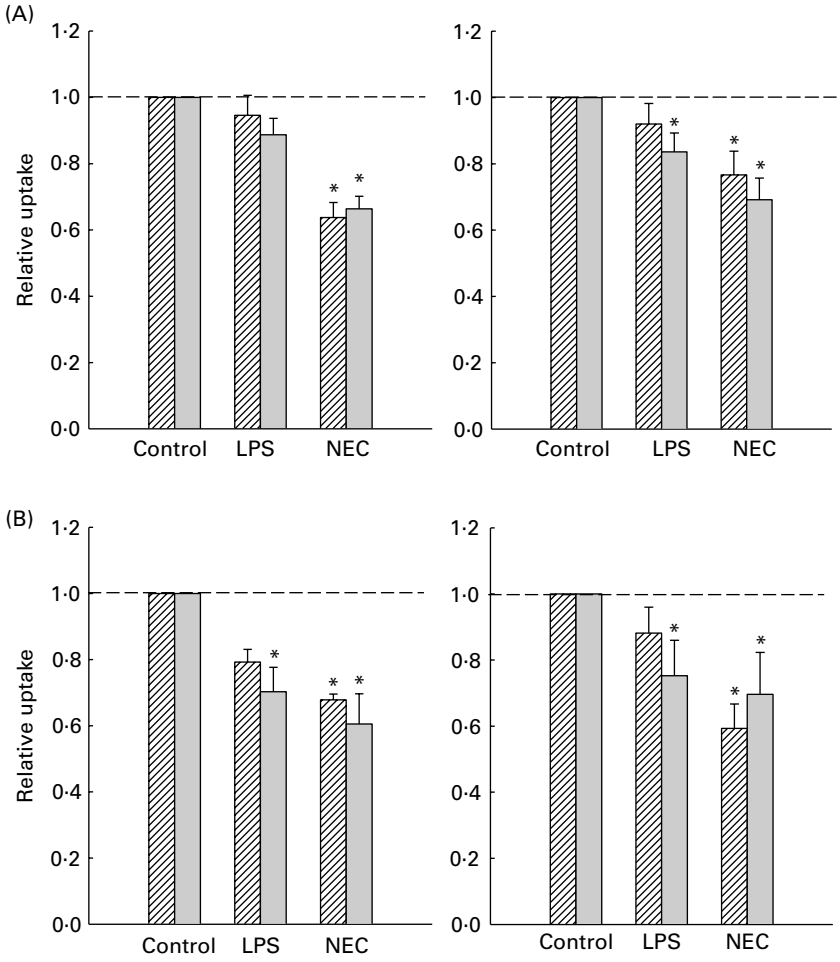

Fig. 3. Relative *ex vivo* uptake of leucine and glucose in tissues stimulated with lipopolysaccharide (LPS) (100 ng/ml) or necrotising enterocolitis (NEC)-microbiota (NEC, 8 × diluted) for 12 min. Tissues were collected from the middle small intestine at birth (▨) or day 2 (▩) in (A) preterm and (B) term pigs. Data are shown as fold change relative to unstimulated tissues at birth and day 2, respectively, and values are means with their standard errors (n 6). * Mean values were significantly different from those of the control group ($P<0{\cdot}05$).

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very well be that other bacteria exert beneficial effects and increase the absorptive functions of the neonate. There is increasing evidence that probiotics may stimulate intestinal health in the healthy premature neonate. It is unclear to which extent probiotics may be harmful in the pro-inflammatory state of extremely low birth weight infants[39]. The important issue seems to be to have the right combination of dietary and bacterial influences to enhance maturation and avoid detrimental effects. We have previously shown that increasing NEC severity leads to a further reduction in nutrient absorptive capacity[28].

Intestinal expression of the pro-inflammatory cytokines, IL-6 and TNF-α, has earlier been shown to be induced in response to bacterial exposure to the developing intestine[21,22]. We showed an increased expression of IL-6 and TNF-α after stimulation with either LPS or NEC-microbiota at birth but the pattern differed between the two cytokines, and at 2 d the inflammatory response was reduced. Our data support the hypothesis that the intestine acquires LPS tolerance after birth by exposure to exogenous endotoxins with feeding and natural colonisation[20]. We were, however, not capable of showing that acquisition of tolerance is less pronounced in preterm neonates, as suggested earlier[19]. Nevertheless, IL-6 mRNA was particularly low in term pigs and not increased at birth or after 2 d of feeding, indicating that this cytokine may be responsive mainly in preterm neonates. The ratio of increase in IL-6 in preterm pigs was 2·2- and 4·6-fold in LPS and NEC-microbiota stimulated tissues, respectively. These increases were much lower than those of IL-8 (78 %) observed in the very immature human fetal intestine (18–21 weeks gestation) stimulated with LPS[19]. Caution is required when extrapolating the results from very immature intestines to the gestational ages relevant for most NEC-sensitive preterm infants. Further, in contrast to the human fetal intestinal tissues, the tissues used in the present study were collected only from healthy pigs with no clinical complications. We suggest that some key functional and immunological functions of the developing intestine

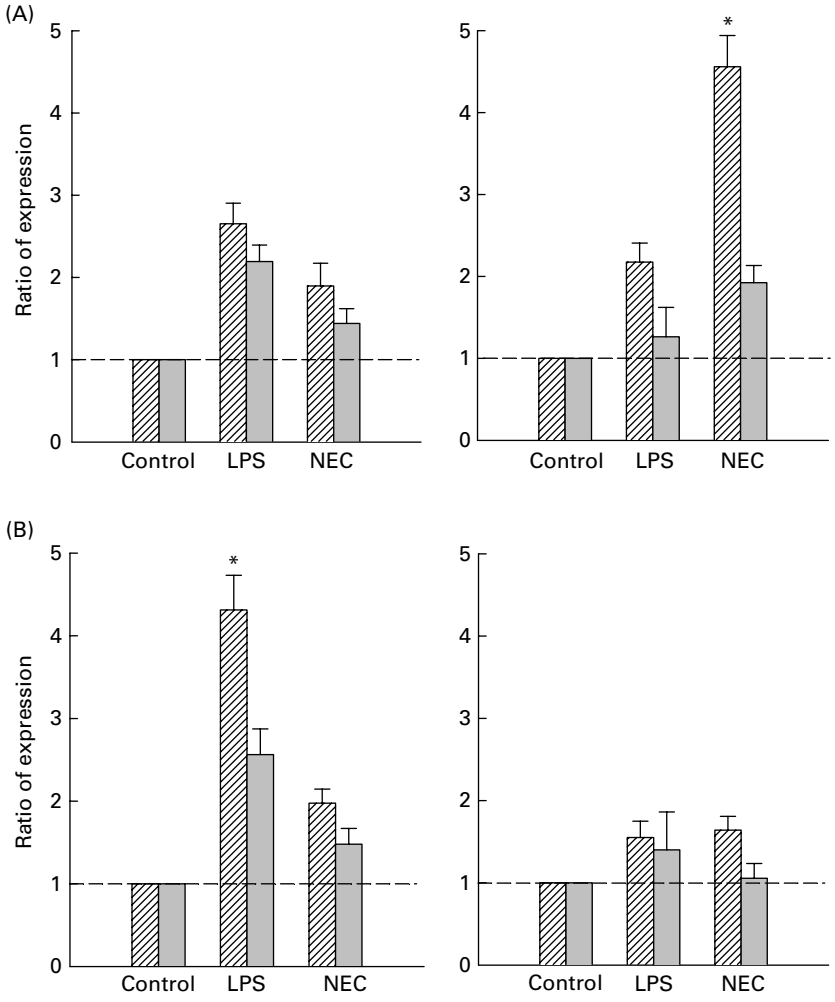

Fig. 4. Relative expression of TNF-α and IL-6 in tissues stimulated *ex vivo* with lipopolysaccharide (LPS) (100 ng/ml) or necrotising enterocolitis (NEC)-microbiota (NEC, 8 × diluted) for 12 min. Tissues were collected from the middle small intestine at birth (▨) or day 2 (□) in (A) preterm and (B) term pigs. Data are shown as fold change relative to unstimulated tissues at birth and day 2, respectively, and values are means with their standard errors (n 6). * Mean values were significantly different from those of the control group ($P<0·05$).

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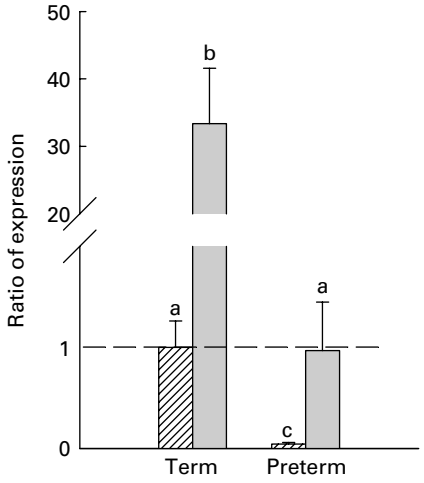

Fig. 5. Relative expression of Toll-like receptor-4 in unstimulated tissues at birth (▨) or day 2 (■) in term and preterm pigs, respectively. Tissues were collected from the middle small intestine. Data are shown as fold change relative to term pigs at birth, and values are means with their standard errors (n 7–8). [a,b,c]Mean values with unlike letters were significantly different between groups ($P<0·05$).

are not markedly affected by moderate reductions in gestational age at birth.

In our pig model of NEC, spontaneous development of NEC occurs after deliveries from 90–95 % gestation, while fetuses delivered before 85 % gestation are barely viable, even with extensive clinical interventions[35]. Our data indicate that the preterm intestine has a remarkably high capacity for rapid catch-up growth and functional adaptation in the days just after birth, and at least *ex vivo*, the intestinal sensitivity to endotoxins is not higher than in term newborns. Lotz *et al.*[20] demonstrated that LPS stimulation of fetal murine IEC *ex vivo* readily resulted in intracellular cell signalling, transcriptional activation and chemokine synthesis and secretion, whereas IEC from newborn and adult mice were non-responsive. Expression of macrophage inflammatory protein 2 reached a maximum 2 h after birth and normalised within 6 h. This loss of susceptibility to LPS stimulation immediately after birth, supported by an early loss of responsiveness (6 h) to a second stimuli *in vitro*[17], would suggest that the *ex vivo* stimulation of intestines from pigs at birth may already have been done in a state of relatively high tolerance to endotoxins. This would explain the lack of consistency for IL-6 and TNF-α gene expressions, both for the short-term stimulation of intact tissues (12 min) and the more long-term stimulation of primary pig IEC (3 h).

TLR-4 is an important pattern recognition receptor for LPS. Previous studies have shown that the small intestine in pigs at birth does express TLR-4[40], as well as it does in human subjects[17], and studies of bacterial recognition by TLR in experimental NEC have demonstrated increased TLR-4 expression in the intestine during NEC[4,41]. The role of TLR-4 signalling in innate epithelial immune tolerance was recently characterised in rodents[42], but the pathways as well as their developmental regulation may differ among species. It is unclear if increased TLR-4 expression is an effect of disease progression or a generally higher basal expression of this receptor in the premature neonate. Our data demonstrate that the expression of TLR-4 follows a developmental pattern and is not increased in the preterm intestinal tissue of otherwise healthy pigs not showing signs of NEC. In our study, TLR-4 expression was the highest at a developmental stage when NEC-sensitivity is minimal, e.g. term pigs after 2 d of feeding and natural colonisation. This suggests that an NEC-related increase in TLR-4 expression is an effect of the disease progression and not a predisposing factor for NEC in the preterm neonate. A previous study in pigs also showed that TLR-4 expression was increased after colostrum feeding of pigs, whereas prenatal LPS treatment failed to increase TLR-4 expression and showed only modest maturation of immunity and NEC resistance[43]. The increase in TLR-4 expression did not account for differences in bacterial tissue responsiveness such as, e.g. leucine and glucose uptake. This indicates that the gene expression level of TLR-4 is not a good indicator for Gram-negative responsiveness. It may well be that post-translational modifications play a significant role for TLR-4, or that expression of the associated molecules MD-2 and CD14 was not equally regulated. Another explanation would be that several other components in the intestine are of importance for the induced sensitivity. Cross-desensitisation among TLR-2 and TLR-4 ligands may be a mechanism to suppress pro-inflammatory responses after repeated contact with Gram-positive or Gram-negative bacteria[17]. This may also explain why the NEC-microbiota, containing a complex mixture of many different bacterial ligands relevant for NEC in pigs, tended to be more effective than LPS. Regardless, it remains that the inflammatory cytokine responses observed in newborn and 2-d-old preterm and term pigs were limited, relative to the reports in other species at other developmental time points[19,42]. Hence, a hyper-responsiveness to Gram-negative endotoxin is less likely to be a main determinant of the increased sensitivity to NEC, at least in preterm pigs. Further studies are required to investigate how the epithelial intracellular pathways may act to control the development of tolerance to bacterial colonisation in the different species just after birth.

Acknowledgements

The present research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors. The authors have no conflicts of interest. Together, S. B. B. and P. T. S. contributed to the study concept and design, acquisition of data, data analysis and interpretation, manuscript preparation and critical comments. S. B. and K. Z. contributed to the data acquisition and analysis. All authors accepted the final manuscript. The authors gratefully acknowledge the scientific input from Lianqiang Che, and thank Kristina Møller and Elin Skytte from Department of Human Nutrition, University of Copenhagen, for their skilful technical support.

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