Background and aims: Necrotising enterocolitis (NEC) is a potentially devastating disorder of preterm infants but its aetiology remains unclear. The aim of these studies was to develop a neonatal piglet model for NEC and to then use the model to investigate the role of platelet activating factor (PAF) in its pathogenesis.

Methods: Anaesthetised newborn piglets were divided into six groups: (i) controls, and groups subjected to (ii) hypoxia, (iii) lipopolysaccharide (LPS), (iv) hypoxia+LPS, (v) hypoxia+LPS and the PAF antagonist WEB 2170, and (vi) PAF. Arterial blood pressure (ABP), superior mesenteric artery blood flow (MBF), mesenteric vascular conductance (MVC), and arterial blood gases were recorded, and intestinal histology was evaluated.

Results: Exposure to LPS, hypoxia+LPS, or PAF all caused haemorrhagic intestinal lesions associated with varying degrees of intestinal injury. PAF caused a significant initial decrease in both MVC and MBF whereas hypoxia+LPS caused a significant late reduction in ABP and MBF with a trend towards a decrease in MVC. The effects of hypoxia+LPS on both haemodynamic changes and intestinal injury were ameliorated by WEB 2170.

Conclusions: Administration of hypoxia and LPS or of PAF in the neonatal piglet induces haemodynamic changes and intestinal lesions that are consistent with NEC. These effects are ameliorated by prior administration of WEB 2170, indicating an important role for PAF in the pathogenesis of NEC.

Abbreviations: NEC, necrotising enterocolitis; PAF, platelet activating factor; LPS, lipopolysaccharide; ABP, arterial blood pressure; PFA, porcine serum albumin; MBF, mesenteric blood flow; MVC, mesenteric vascular conductance; SMA, superior mesenteric artery; WBC, white blood cell
The aim of the present study was therefore to develop a model for NEC in the neonatal piglet and to investigate the potential role of PAF in its pathogenesis.

METHODS

Animals

Twenty seven full term neonatal Canberra White piglets were used. Their median (range) age at the time of study was 3 days (2–6) and weight 1997 g (1465–2270). All experiments were conducted according to the Home Office (UK) Animal (Scientific Procedures) Act (1986). Animals were nursed normally by the sow until 2–3 hours before the beginning of the experiment. In each animal, anaesthesia was induced and maintained as described by Elnazir and colleagues.23

Operative protocol

Briefly, 3–5% halothane (Rhodia Organique Fine Ltd, Avonmouth, UK) in oxygen was delivered at 3.5 l/min initially into an anaesthetic box and then via a facemask. After induction of anaesthesia, the animal was intubated and anaesthesia was maintained with 2.5% halothane in O2 while the jugular vein was cannulated. Inhalation anaesthesia was then discontinued and anaesthesia was maintained with an intravenous infusion of Saffan (Pitman-Moore, Uxbridge, UK). Initially, a priming dose of 4–8 mg/kg Saffan diluted with normal saline to 4 mg/ml was given in small boluses. Then, Saffan was given in the same concentration by continuous intravenous infusion at 16–24 mg/kg/h during surgery and at 7–12 mg/kg/h during the experimental period. Saffan is a short acting steroid agent that can be given as a continuous infusion so as to achieve a stable level of anaesthesia with analgesia. This agent was chosen because it preserves central neural control of the cardiovascular system more effectively than other commonly used anaesthetics.24 Once anaesthesia was established and throughout the experiment, rectal temperature was continuously monitored and maintained at 38.5–39.5 °C by means of heat pads beneath and heat lamps above the animal.

Respiratory variables were recorded by means of a respiratory flow head attached to the end of the endotracheal tube and connected to an electrospirometer allowing respiratory frequency and tidal volume to be recorded continuously. Pulsatile arterial blood pressure (ABP) was recorded from a cannula in a femoral artery and thereby heart rate was derived. A femoral vein was also cannulated and the catheter advanced into the inferior vena cava to allow central venous administration of drugs.

A laparotomy was then performed via a midline incision. A branch of the cranial (superior) mesenteric artery (SMA) was identified and the SMA was isolated by careful dissection. A 5 or 6 mm circumference cuff-type electromagnetic flow probe (Carolina Systems Inc., King, North Carolina, USA) was then placed around the SMA for continuous recordings of mesenteric blood flow (MBF). The flow probe was zeroed by temporarily occluding the vessel. Bowel handling was kept to a minimum and exposed bowel was covered in gauze swabs soaked in warm (38°C) saline. Once the flow probe was in situ, the bowel was returned to the abdominal cavity and the incision closed. Following abdominal closure, a stabilisation period of 60 minutes took place.

All physiological variables were sampled at 40 Hz and acquired onto a PowerMac 4400/160 by a MacLab/8e System (AD instruments Ltd, Oxon, UK). Mesenteric vascular conductance (MVC) was calculated in real time by division of MBF by ABP.

Experimental protocols

The animals were divided into six groups. The experimental protocol commenced after the stabilisation period when all physiological recordings were stable and comprised three 45 minute periods.

Development of model for NEC

Group 1 (control group; n = 4) spontaneously breathed room air throughout and received an intravenous bolus injection of 1 ml/kg of porcine serum albumin (PSA) via a central vein at the start of the experimental period (T0).

Group 2 (hypoxia group; n = 3). These animals breathed room air for the first 45 minutes from T0. This was followed by a 45 minute period of breathing 10% oxygen and then by a 45 minute period breathing room air.

Group 3 (endotoxin (lipopolysaccharide (LPS)) group; n = 4) spontaneously breathed room air throughout and received a bolus of LPS (2 mg/kg) via the femoral vein at T0.

Group 4 (hypoxia,endotoxin group (hypoxia+LPS); n = 6) followed the same protocol as group 2 but also received a dose of LPS at T0 as in group 3.

The role of PAF

Group 5 (hypoxia+LPS+WEB group; n = 5) received LPS and hypoxia as in group 4. However, this group received 10 µg/kg of the PAF antagonist WEB 2170 via the femoral vein, 10 minutes prior to T0. This dose was chosen based on previously described therapeutic activity25 and following preliminary experimentation.

Group 6 (PAF group; n = 5) spontaneously breathed room air throughout and received an intravenous bolus injection of 5–7.5 µg/kg PAF at T0. The dose range for PAF chosen was comparable with that used in the rat model of NEC.11 The first animal received 5 µg/kg and the next four animals received 7.5 µg/kg.

Blood and tissue sampling

Arterial blood samples were taken at T0 and at regular intervals throughout the experiment. These were analysed for pH and arterial PO2 and PCO2 (Pao2 and Paco2, respectively) using an IL1640 analyser (Instrumentation Laboratories, Warrington, Cheshire, UK) and for peripheral blood count using a MaxM Coulter counter (Beckman Coulter, USA). All experiments were terminated 135 minutes after T0 and the animals were killed by a lethal dose of pentobarbitone sodium. The thoracic and abdominal organs were removed immediately. The lumen of the gastrointestinal tract from the oesophagus to the rectum was perfused with 10% neutral buffered formalin, and then all organs placed in buffered formalin until pathological examination was performed. After fixation, the bowel was examined externally for gross lesions, particularly petechial or frank haemorrhage. Serosal lesions were identified and measured. Tissue samples were taken by one pathologist: biopsies of the serosal lesions were taken and serial biopsies were also taken at 10–15 cm intervals throughout the small and large bowel. In all cases, samples were also taken from the liver, kidneys, lungs, heart, and stomach. Sections of 4 µm thickness were cut and stained with haematoxylin and eosin.

Sections were then examined by two pathologists who were blinded to the study group. Histological assessment of the most severe intestinal injury was made in each case and given a score of 0–4: 0—no significant abnormality; 1—mild abnormality; 2—moderate abnormality; 3—severe abnormality (localised); and 4—severe abnormality (generalised) (see table 1).
Details of drug preparation
(i) PSA was made up fresh with saline on the morning of the experiment to a concentration of 2.5 mg/ml and was stored on ice until use.
(ii) PAF (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine; Sigma-Aldrich, Gillingham, UK) was made up from the lyophilised form with ethanol to a concentration of 2 mg/ml. This solution was then divided into 100 µl aliquots (50 µl) and stored at −70°C in Eppendorf tubes. On the day of the experiment, fresh solutions of PAF were made up with PSA after thawing and drying the PAF in nitrogen gas. Prior to use, PAF was dissolved in PSA to a concentration of 7.5 µg/ml.
(iii) LPS from Escherichia coli serotype 026:B6 (Sigma-Aldrich) was made up to a concentration of 4 mg/ml by dissolving in freshly prepared saline.
(iv) WEB 2170 (Bepafant; 5-(2-chloro-phenyl)-3,4-dihydro-2H, 7H-10-methyl-3-[(4-morpholinyl) carbonyl]-[4,5]thieno[3,2-f][1,2,4]triazolo-[4,3-e][1,4]diazepine)] (a generous gift from Bayer AG, Leverkusen, Germany) was diluted to 60 µg/ml in sterile deionised water. All solutions were stored on ice until used and were brought to room temperature before injection.

Statistical analyses
All physiological data were subsequently analysed by taking the mean of 30 second samples of the data recorded on the MacLab at regular intervals, and are expressed as mean (SEM). Data from each experimental group were analysed by repeated measures and factorial ANOVA with Bonferroni post hoc tests using the Statview computer software package (version 5; SAS Institute, Cary, North Carolina, USA) in order to test differences from baseline and between groups, respectively. In all cases, p values of <0.05 were taken to be significant.

RESULTS
The laparotomy was well tolerated by all animals. Physiological variables remained stable during the surgical procedure and the subsequent 60 minute postoperative stabilisation period.

Haemodynamic variables
In the control group (group 1), all physiological variables recorded remained stable over the experimental period (see figs 1, 2). In the other groups, the following were noted.

Blood pressure
In the hypoxia group (group 2), there was no change in blood pressure throughout the experiment. However, in the group exposed to LPS (groups 3), there was a significant fall in mean ABP at 45 and 90 minutes (p<0.01) but this recovered to baseline by the end of the experiment. In the hypoxia+LPS group (group 4), there was a moderate but non-significant fall in ABP towards the end of the experiment (see fig 1) but in the hypoxia+LPS+WEB group (group 5), mean ABP did not alter significantly. In the PAF group (group 6), there was a brief period of hypotension (lasting only 30 seconds) immediately after administration of PAF. Brief bradycardia and apnoea were also noted over this time period.

Mesenteric blood flow
In the hypoxia and LPS group (groups 2 and 3), there was no significant change in MBF. By contrast, MBF fell steadily in the hypoxia+LPS group (group 4) and was significantly reduced at 90 and 135 minutes (p<0.01 and p<0.001, respectively). However, in the hypoxia+LPS+WEB group (group 5), there was no significant change in MBF (fig 1).

In the PAF group (group 6), there was an immediate fall in MBF following injection of PAF and this remained significantly depressed at approximately 30% of baseline for at least 15 minutes (p<0.001), gradually returning to baseline over the following 50 minutes. The fall in MBF is attributable to a decrease in MVC (see fig 1).

Blood gas analysis
Arterial blood gas analysis (P aO2, P aCO2, and pH) remained stable in the control group throughout the experiment.

All groups subjected to hypoxia (groups 2, 4, and 5) developed similar degrees of hypoxaemia during the 45 minute period of breathing 10% oxygen. In these groups, P aO2 fell from 68 (2.0) mm Hg at the start of the experiment to 40 (2.0) mm Hg at 90 minutes; mean P aO2 in these groups recovered to 68 (6.3) mm Hg after the period of hypoxia. There was no significant difference in P aO2 measurements between these hypoxic groups at any time during the experiment.

P aCO2 measurements in all groups remained essentially stable throughout the experiment with no significant difference between groups at any time.

Baseline pH in all groups was 7.40 (0.01). In the control and hypoxia groups (groups 1 and 2), pH tended to increase towards the end of the experiment (fig 2).

There was a non-significant fall in pH in the LPS group (group 3) but in the hypoxia+LPS group (group 4) there was a fall in mean pH, such that pH was significantly lower than controls at 90 minutes (p<0.01) but recovered slightly at the end of the experiment. This effect was ameliorated to some extent in the hypoxia+LPS+WEB group (group 5, fig 2). The PAF group (group 6) developed a metabolic acidosis after 15 minutes, with a fall in mean pH from 7.43 (0.01) to 7.29 (0.04) (p<0.01) which was sustained until 45 minutes but recovered by 90 minutes (fig 2).

Haematological analyses
Compared with controls, over the duration of the experiment the following results were obtained: (i) there was no significant change in haemoglobin in any of the groups; (ii) there was a significant fall in white blood cell (WBC) count in the groups who received endotoxin (groups 3, 4, and 5; p<0.001) but no significant change in the hypoxia and PAF groups (groups 2 and 6). The observed fall in WBC occurred as a result of neutropenia; (iii) there was a modest but statistically significant fall in the platelet count for groups 2, 3, 4, and 5 (p<0.05) but not for the PAF group (group 6).
platelet count was never $<350 \times 10^9/l$ in any of the groups at any time.

**Pathological analyses**

Macroscopic and microscopic examination showed that the bowel from both the control group and the hypoxia group (groups 1 and 2) were normal (table 2). Macroscopic examination of the LPS, hypoxia+LPS, and PAF groups (groups 3, 4, and 6) revealed that when compared with controls, the serosal surface of the small and large intestine had a uniform dusky discolouration, and scattered haemorrhagic lesions from 0.1 to 10 cm in length were present: the majority were localised to the ileum and caecum. Microscopic examination of intestine from these groups revealed a spectrum of the pathological features described in table 1. Lesions were more severe in the PAF group and included evidence of transmural haemorrhagic necrosis. When comparing bowel injury in groups 3 and 4, LPS alone tended to cause discrete localised haemorrhagic lesions whereas hypoxia+LPS resulted in more widespread haemorrhage. There was no evidence of thrombus formation in any of the specimens.

WEB 2170 had a powerful protective effect with respect to the effects of hypoxia+LPS. In contrast with the marked

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**Figure 1** Physiological parameters for the study groups. (A) Arterial blood pressure (ABP); (B) mesenteric blood flow (MBF); and (C) mesenteric vascular conductance (MVC). Significant difference between the value recorded and the control group: **p<0.01, ***p<0.001. Significant difference between the value recorded and the baseline value for that group: **p<0.01, ***p<0.001. PAF, platelet activating factor; LPS, lipopolysaccharide; WEB, WEB 2170.
The role of PAF in necrotising enterocolitis

Records and the baseline value for that group: WEB WEB 2170.

Difference between the value recorded and the control group:

DISCUSSION

This study has demonstrated that in the neonatal piglet, the combined insults of hypoxia and endotoxin induced a macroscopic and histological changes seen in the hypoxia+LPS group, the surface of the gut appeared normal in the hypoxia+LPS+WEB group. Indeed, all but one animal in the hypoxia+LPS+WEB group (group 5) had normal intestinal histology. In this exceptional animal there was a single petechial haemorrhage <1 mm in diameter—the rest of the bowel was normal.

Sections of the other major organs (heart, lungs, liver, and kidneys) were also examined microscopically and no abnormality was found in any group.

Our finding that the haemodynamic responses, the metabolic acidosis, and the NEC-like lesions induced by hypoxia+LPS could be ameliorated by prophylactic administration of the PAF receptor antagonist WEB 2170, provides compelling evidence that PAF is causally involved in NEC. It is consistent with evidence obtained in adult rats that systemic hypoxia or endotoxin or a combination of the two can lead to endogenous PAF production. The combined insult of hypoxia plus LPS has been shown to act synergistically in rats with respect to hypotension, fall in MBF, and endogenous PAF production. This is entirely consistent with our findings.

The proposal that PAF is a causal mediator in the effects of hypoxia plus LPS is supported by our finding that administration of exogenous PAF to the neonatal piglet also produced haemodynamic changes and intestinal lesions similar to the combination of hypoxia plus LPS. However, it should be noted that gross systemic hypotension and a fall in MBF with macroscopic and microscopic evidence of intestinal lesions. However, combining hypoxia with LPS markedly accentuated the hemodynamic changes, such that arterial pressure and MBF fell progressively, due in part to mesenteric vasoconstriction, and there were more widespread intestinal lesions than seen with LPS alone. In addition, this combined insult produced a significant metabolic acidosis during the experiment which may have occurred as a result of gut ischaemia. This pattern of responses seems to mimic very closely the pathophysiological process that may occur in early NEC in the human neonate. It should be noted that gross systemic hypotension is not a feature of the early stages of human NEC. These findings are fully consistent with the clinical experience that NEC is more likely when more than one of the common risk factors for NEC occur in concert than when singly.

It has been reported previously that intestinal lesions similar to those of NEC can be produced in the piglet only by gross insults: severe ischaemia involving ligation or occlusion of mesenteric arteries; profound asphyxia; or intraluminal injection of acidified casein. None of these experimental insults reflect conditions that occur in the clinical setting. As far as we are aware, ours is the first study to show that NEC-like lesions can be produced in the neonatal piglet by combining the much more clinically plausible insults of moderate hypoxia with endotoxaemia.

Our finding that the haemodynamic changes, the metabolic acidosis, and the NEC-like lesions induced by hypoxia+LPS could be ameliorated by prophylactic administration of the PAF receptor antagonist WEB 2170, provides compelling evidence that PAF is causally involved in NEC. It is consistent with evidence obtained in adult rats that systemic hypoxia or endotoxin or a combination of the two can lead to endogenous PAF production. The combined insult of hypoxia plus LPS has been shown to act synergistically in rats with respect to hypotension, fall in MBF, and endogenous PAF production. This is entirely consistent with our findings.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Details of the experimental groups and degree of intestinal injury. The histological grade in each case is the most severe lesion seen.</th>
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<td>Group</td>
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<td>Control</td>
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<td>Hypoxia (H)</td>
<td>3</td>
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<tr>
<td>LPS</td>
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<td>PAF</td>
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PAF, platelet activating factor; LPS, lipopolysaccharide; WEB, WEB 2170.
those of NEC. The fact that these haemodynamic responses were of a different time course and more substantial, comprising an earlier and more dramatic mesenteric vasoconstriction, and that the intestinal lesions were more severe than those induced by hypoxia plus LPS, is not unexpected. Injection of a large dose of PAF into the systemic circulation is clearly very different from the likely profile of endogenous PAF release from body tissues. Nevertheless, the observation that exogenous PAF induced strong mesenteric vasoconstriction per se, as when the SMA of the piglet was completely included for 60 minutes, this resulted in detectable bowel lesions in the mucosa and submucosa, but not in the muscularis or serosal layers, as we observed in response to PAF.

Given that PAF has a short half life in vivo, it is probable that the exogenous PAF we administered stimulated endogenous PAF production via a positive feedback loop and that the exogenous PAF we administered stimulated the production of further inflammatory mediators. The combined effect of all of these mediators would then explain the development of the more severe intestinal necrosis with features characteristic of the later stages of NEC. We now hypothesise that if the intestinal lesions induced by hypoxia plus LPS were initiated by endogenous PAF as we have proposed, the whole process would then have been fuelled by the influence of further PAF generated by positive feedback and other cytokines. According to this hypothesis, the extent of the intestinal lesions and the severity of the accompanying haemodynamic changes would depend on the severity of the precipitating insults, the magnitude of the increase in PAF generated, the time that elapses between the insults, and the time of observation, and also on natural inhibitors such as PAF acetylhydrolase that are available to limit the pathological cascade.

NEC is not seen in adults and very rarely seen in older infants: the neonate, particularly if born prematurely, appears to be uniquely susceptible to the disease. The gut vasculature of the newborn piglet is less well able to preserve endogenous PAF production via a positive feedback loop and that the exogenous PAF we administered stimulated endogenous PAF production via a positive feedback loop and that the exogenous PAF we administered stimulated endogenous PAF production via a positive feedback loop and that the exogenous PAF we administered stimulated endogenous PAF production via a positive feedback loop and that the exogenous PAF we administered stimulated endog...

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The role of platelet activating factor in a neonatal piglet model of necrotising enterocolitis
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