

Relationship between tissue hypoxia and apoptosis: a preliminary observational study

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Perturbations in oxygen delivery (hypoxia and hypotension) to, and consumption by, tissue are common in critical illness. Under these conditions, neurohumoral reflexes promote cutaneous and splanchnic vasoconstriction and redistribute perfusion to ensure myocardial and cerebral oxygenation. However, the trade-off is splanchnic and subcutaneous tissue hypoxia and hypercapnia, which have been demonstrated in previously well established models of haemorrhagic¹ and endotoxic² shock. We have also shown that, if severe and persistent, mucosal hypoxia and hypercapnia can lead to significant epithelial dysfunction, such as altered permeability³ and protein-losing enteropathy.⁴ At a molecular level, it is well recognised that deprivation of oxygen to the tissues results in tissue dysoxia, as evidenced by generation of lactate in the tissues and reductions in cellular ATP concentrations.

More recently, attention has focused on the ability of hypoxic stress to induce apoptosis.^{5,6} Apoptosis of the gut mucosa in critical illness has been demonstrated by Hotchkiss et al even after brief duration of shock.⁷ Shock, trauma and ischaemia–reperfusion were thought to be the inciting factors in this study. Other investigators have also demonstrated the link between tissue hypoxia and apoptosis.^{8–11} This finding is important as it is now believed that apoptosis of a variety of cells might contribute to the morbidity in critical illness. Altered immune cell apoptosis is thought to underlie some of the deleterious manifestations of sepsis.¹² Endothelial cell apoptosis may play an important role in the genesis of sepsis-induced multiple organ dysfunction syndrome.¹³ Coopersmith et al have demonstrated that inhibition of apoptosis had survival benefit in mice subjected to sepsis from caecal ligation and puncture.¹⁴ Supportive evidence for the detrimental effects of apoptosis also comes from studies showing that inhibition of caspase, an integral enzyme in the apoptotic pathway, improves outcome in sepsis.¹⁴

While apoptosis is an accepted consequence of tissue hypoxia, the precise relationship between severity of hypoxia and apoptosis has not been clearly established.^{5,6,8–11} In most studies demonstrating the link between the two, cell cultures from animal models were incubated under extreme hypoxia or total anoxia, in some cases for prolonged periods.^{5,6,8,10,11} Varying degrees of apoptosis were demonstrated in these experiments. While these studies elucidated important pathophysiological aspects of

ABSTRACT

Aims: This observational study examined the relationship between tissue hypoxia (PO₂ of 30 mmHg and 15 mmHg) and the development of dysoxia and apoptosis.

Methods: 28 Sprague–Dawley rats were studied in three groups. Group 1 had no interventions (controls; *n* = 6). Graded hypoxia was induced in Group 2 (*n* = 13) and Group 3 (*n* = 9) to achieve a subcutaneous PO₂ of < 30 mmHg (20 minutes) followed by < 15 mmHg (20 minutes). In addition, Group 3 received reoxygenation to baseline after hypoxia. Ileal and cutaneous tissues were assessed for apoptosis (by histology, TUNEL and immunohistochemistry for caspase-3) and dysoxia (tissue lactate concentration and energy charge).

Results: An interstitial PO₂ < 30 mmHg led to statistically significant elevations in skin and gut lactate concentrations from baseline (mean ± SD: skin, 0.2 ± 0.07 to 0.6 ± 0.3 mmol/kg wet weight, *P* < 0.01; gut, 1 ± 0.7 to 6 ± 4 mmol/kg wet weight, *P* < 0.05). With hypoxia, there was an increase in apoptosis scores in the gut villi from baseline in the experimental arms (17% ± 21% to 53% ± 58%, *P* < 0.05 in Group 2; 9% ± 5% to 27% ± 12%, *P* < 0.05 in Group 3). There was no significant increase in skin apoptosis. No significant correlation was noted between gut lactate concentrations and gut apoptosis (*r* = -0.28).

Conclusions: In this pilot study, reductions in PO₂ to < 30 mmHg were associated with significant dysoxic changes in the gut and skin. No clear tissue PO₂ threshold for the initiation of apoptosis was identified. Further studies with refinements to the experimental model may allow more precise identification of PO₂ thresholds that are critical for the development of apoptosis.

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hypoxia-induced apoptosis, their results cannot be directly extrapolated to critical illness, as both the severity and duration of imposed hypoxia were incompatible with life.

We therefore investigated whether reductions in tissue PO₂ down to a level of 30 mmHg would trigger apoptotic changes in the tissues. We also investigated whether further reductions in PO₂ down to 15 mmHg would gener-

ate incremental histological change. We chose the 30 mmHg threshold of tissue PO₂ because of our observations in a previous animal study of haemorrhagic shock that the nadir subcutaneous tissue PO₂ was close to 30 mmHg at the height of shock.¹ Furthermore, the normal end-capillary venous oxygen tension, a close approximation of tissue PO₂, is about 37.5 mmHg.¹⁵ Thus, perturbations in oxygen delivery even of small magnitude can result in reductions in tissue PO₂ to below 30 mmHg.

We therefore undertook a laboratory study to examine:

- whether reductions in tissue PO₂ below 30 mmHg result in apoptosis; and
- whether the changes of tissue dysoxia and cellular changes of apoptosis are temporally related.

Methods

The study was conducted in Sprague–Dawley rats and was approved by the University of Queensland Animal Experimentation Ethics Committee.

Animal model

All rats were anaesthetised with intraperitoneal sodium pentobarbital (60 mg/kg) and ventilated via a tracheostomy with supplemental oxygen and isoflurane using a Harvard Rodent Ventilator 683 (South Natick, Mass, USA). Heat loss was reduced by placing the anaesthetised rats on a warming pad under reflecting metal foil. A 20-gauge cannula was placed in the left carotid artery via a small neck incision, and the mean arterial blood pressure (MAP) was monitored continuously using a pressure transducer (Model 43-212, Baxter Edwards Critical-Care, Irvine, Cal, USA), with the output displayed on a portable monitor (1275A, Hewlett-Packard, Waltham, Mass, USA). MAP was recorded at 1-minute intervals throughout the experiment. Minute ventilation was adjusted until the PaCO₂ was in the range 40–60 mmHg as determined by blood gas analysis (ABL 725, Radiometer, Copenhagen, Denmark). Physiological saline was infused into the carotid artery at a rate of 3 mL/h. The inspired isoflurane concentration was adjusted to maintain surgical anaesthesia, with an MAP target of 100 mmHg. A calibrated Paratrend 7 sensor (see below) was inserted into the subcutaneous tissue of the ventrolateral torso from the inguinal area to the axilla for a minimum of 8 cm to measure tissue gas tensions.

Before the study began, the subcutaneous site was inspected to confirm the absence of tissue damage. Steady-state conditions were defined as those during which the animal remained haemodynamically stable (< 10% change in blood pressure from baseline) without intervention, a stable tissue PO₂ (< 10% change in tissue PO₂), and a temperature within the set range.

The rats were studied in three groups: Group 1 (control arm), Group 2 (hypoxic hypoxia arm) and Group 3 (hypoxia–reoxygenation arm).

Experimental procedure

Group 2 (hypoxic hypoxia arm): After establishing steady-state conditions, baseline tissue gas tensions, and arterial plasma pH and lactate measurements were recorded. Tissue specimens were collected for measurement of lactate and nucleotide concentrations and quantification of apoptosis. Hypoxia was induced by progressive decrement in inspired oxygen concentration (using supplemental nitrogen in the inspiratory limb). The F_{IO₂} was titrated to achieve two levels of tissue hypoxia in the subcutaneous tissue: Level 1, tissue PO₂ of 15–30 mmHg; and Level 2, tissue PO₂ of 0–15 mmHg.

Each level of hypoxia was maintained for 20 minutes. Tissue gas tensions were measured, and simultaneously specimens of the subcutaneous and ileal tissue were collected for tissue nucleotide and lactate analysis and apoptosis quantification.

Group 3 (hypoxia–reoxygenation arm): The experiment was performed as above with the addition of two reoxygenation steps. Following the second (Level 2) hypoxic period, inspired oxygen concentration was progressively stepped up to return to Level 1 hypoxia for 20 minutes, followed by an increase in F_{IO₂} to return tissue PO₂ to baseline. Following 20 minutes of ventilation at each new inspired oxygen concentration, tissue gas tensions were measured, and specimens of the subcutaneous and ileal tissue were collected for tissue nucleotide and lactate analysis and apoptosis quantification.

Group 1 (control arm): Dissection and monitoring were the same as for the other groups. However, there were no changes to F_{IO₂}. Sampling for lactate, nucleotides and apoptosis were carried out at the same time points as for the other groups: Times 1 (baseline), 2 (after Level 1 hypoxia), 3 (after Level 2 hypoxia), 4 (after reoxygenation to Level 1 hypoxia) and 5 (after reoxygenation to baseline).

As Group 2 did not go through the reoxygenation part of the experiment, there were no data points for this group corresponding to Times 4 and 5.

At the end of the study period, all rats were killed under anaesthesia by bilateral thoracotomy.

Paratrend 7 sensor

The Paratrend 7 (Diametrics Medical, Bucks, UK) is a multiparameter sensor, comprising optodes for the measurement of pH, PCO₂ and PO₂, and a thermocouple for the measurement of temperature.^{16–18} We have demonstrated its ability to measure tissue gas tensions previously.^{1,2} Tissue gas tensions were recorded every 10 seconds and trans-

ferred as an ASCII file through an RS232 port to the Windows 3.1 Terminal Application (Microsoft, Redmond, Wash, USA) on a laptop computer.

Quantification of apoptosis

Tissue collection: At indicated times, sections of ileum and subcutaneous tissue were rapidly removed, fixed in 4% buffered paraformaldehyde (pH, 7.4) at 4°C overnight, then transferred to 70% buffered ethanol before preparation for histological and immunohistochemical examination. Tissue was blocked routinely in paraffin, and 4 µm sections were cut onto Superfrost Plus slides for staining. Histological stains included haematoxylin and eosin, periodic acid–Schiff reagent for protein localisation, and Masson's trichrome stain for collagen.

Apoptosis: Previously defined morphological criteria were used to count apoptotic cells.¹⁹ These included cellular rounding and shrinkage, eosinophilic cytoplasm, nuclear chromatin compaction, especially along the nuclear envelope in a crescentic manner, membrane-bound cellular blebbing, and formation of apoptotic bodies which may be phagocytosed by invading macrophages. Apoptosis was counted in at least 10 fields per section (at least 1000 cells) at × 400 microscope magnification. Cells that fell within the 100 squares of an eyepiece graticule were counted. Number of apoptotic cells was expressed as a percentage of total cells counted. TUNEL (terminal dideoxyuridine transferase-mediated nick end labelling) analysis was used as biochemical verification of morphologically assessed apoptosis in skin and gut. The ApoTag Peroxidase In Situ Apoptosis Detection Kit was used according to the manufacturer's protocol for paraffin-embedded sections (Serologicals Corporation, Chemicon, Melbourne, VIC).

Necrosis: This was assessed morphologically¹⁹ by cellular swelling and lysis, cytoplasmic eosinophilia, membrane rupture, densities in swollen mitochondria, pyknotic or irregularly clumped nuclear chromatin, and an associated leukocytic infiltrate which was absent in apoptosis. Other evidence of tissue damage, cytoplasmic blebbing, loss of cellular adhesion, and abnormal but non-necrotic nuclear swelling, was also recorded.

Caspase-3 activation: As added verification of the presence of apoptosis, activated caspase-3 was assessed using immunohistochemistry and methods previously described.²⁰ Both negative and positive controls were included. Cells positive for activated caspase-3 were assessed using microscopy.

Measurement of tissue nucleotides

Adenine nucleotide concentrations in the gut, expressed per gram of tissue protein, were measured using a previ-

ously described method.²¹ Values for the total adenine nucleotides [TAN], calculated as [ATP + ADP + AMP], were expressed per gram of protein. The energy charge (EC) was calculated as [ATP] + [0.5*ADP/TAN].

Tissue lactate analysis

The sample preparation and homogenisation of tissue was performed using a previously described method.²¹ Three reference blanks were included in the assay. The samples were assayed according to the instructions of the BioMerieux PAP kit (cat. no. 61192, BioMerieux, Sydney, NSW), but the volumes were modified for a 96-well plate and measured at 492–550 nm on a 96-well plate reader (ie, sample volume was unchanged at 10 µL, but diluted with 100 µL instead of 1 mL working solution). Standards were first diluted 1/10 with deionised water, and then 10 µL of this was used with 100 µL of working solution to produce the same standard curve as per assay instructions. The standards added corresponded to added amounts of 15, 30 and 60 nmoles lactate. The concentration of lactate in the solution was calculated from the resulting curve.

The lactate content of the sample was calculated from the equation:

Lactate content (in mmol/kg dry weight) = [(180 + K₂CO₃ volume)/1000] × (11.11 × concentration of lactate in solution in nmol/dry weight of homogenised sample in mg).

To convert to wet weight, the samples were divided by their respective rat tissue mean water content ratios (for skin, 2.36; and for gut 2.7). These ratios were predetermined by measuring untreated, wet samples and reweighing when freeze-dried.

Blinding

The investigator quantifying apoptosis was blinded to the tissue PO₂, nucleotide and lactate values. The investigator performing the tissue nucleotide assays was blinded to the tissue PO₂ and lactate values.

Statistical analysis

Analysis was performed by SAS version for Windows 9.1 (SAS Institute, Cary, NC, USA). Analysis of covariance was used to test the effect of experimental group on the dependent variables of interest (lactate concentrations, nucleotide assays and apoptosis scores) at each follow-up time point after adjustment for baseline score. Skin and gut lactate concentrations and apoptosis scores were log-transformed to remove skew before analysis. Spearman's rank method was used to estimate correlations between apoptosis and indices of dysoxia. Statistical significance was defined to be at the conventional 95% level (two-tailed).

Results

Twenty-eight animals were studied, six in Group 1 (control arm), 13 in Group 2 (hypoxia arm) and nine in Group 3 (hypoxia–reoxygenation arm). Eight animals were excluded from the analyses: one from Group 1, five from Group 2 and two from Group 3. Reasons for exclusion were early death from intraperitoneal bleeding and hypoxia during set up of the experiment (three animals), early hypoxia and hypotension before commencement of

experimental interventions (three animals), problems with accuracy of tissue PO₂ measurements, and hence attainment of target PO₂ (one animal), and loss of tissue samples (one animal). Thus, data from 20 animals were available for analysis. Their characteristics at baseline are outlined in Table 1.

Table 1. Comparison of baseline data between the three groups (mean ± SD)

	Group 1	Group 2	Group 3
Weight (g)	413±20	416±18	430±45
MAP (mmHg)	125±21	117±20	124±16
pHa	7.39±0.05	7.37±0.04	7.35±0.04
PaCO ₂ (mmHg)	40±3	40±7	44±6
PaO ₂ (mmHg)	316±21	252±101	305±54
Plasma lactate (mmol/L)	1.8±0.5	2.0±0.7	1.8±0.2

MAP = mean arterial pressure. pHa = arterial pH. ♦

Tissue PO₂

The baseline tissue PO₂ in the subcutaneous tissue was comparable between the three groups, with no significant differences between groups (mean±SD: 74±37 mmHg in controls, 59±7 mmHg in the hypoxia group and 61±15 mmHg in the reoxygenation group; *P*=0.23). Tissue PO₂ levels remained similar to baseline levels in the control group and reached the appropriate target levels in the experimental groups.

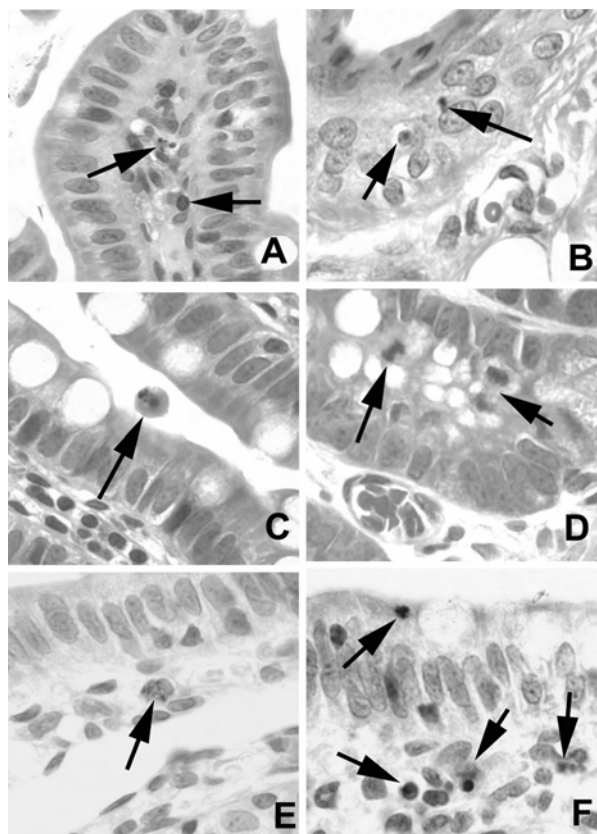
Arterial and tissue lactate concentrations

Plasma lactate: As noted, there were no differences in plasma lactate concentration at baseline (Time 1) between the three groups. Changes in serial arterial plasma, gut and skin lactate concentrations are outlined in Table 2. Arterial plasma lactate concentrations rose significantly

Table 2. Plasma and tissue lactate concentrations, gut apoptosis scores and energy charge (mean ± SD)

	Baseline	Level 1 hypoxia	Level 2 hypoxia	Reoxygenation to level 1 hypoxia	Reoxygenation to baseline
Arterial plasma lactate (mmol/L)					
Group 1 (controls)	1.8±0.6	1.7±0.3	1.3±0.2	1.4±0.5	1.4±0.2
Group 2	2±0.7	3.7±1.4*	4.1±1.8*	na	na
Group 3	1.8±0.2	3.2±0.9*	4.1±1.5*	3.4±1.6	2±0.6
Skin lactate (mmol/kg ww)					
Group 1 (controls)	0.2±0.06	0.2±0.09	0.2±0.2	0.2±0.03	0.1±0.1
Group 2	0.2 ± 0.07	0.6 ± 0.3 [†]	0.6 ± 0.2 [†]	na	na
Group 3	0.1±0.03	0.4 ± 0.2*	0.7 ± 0.4 [†]	0.4±0.2 [†]	0.3±0.2*
Gut lactate (mmol/kg ww)					
Group 1 (controls)	3±2	4±5	2±0.8	3±1	2±1
Group 2	1±0.7	5±3	6±4*	na	na
Group 3	1±0.6	4±4	5±2*	3±2	1±1
Gut apoptosis score (%)					
Group 1 (controls)	8±8	17±11	21±10	23±9 [‡]	25±19
Group 2	17±21	31±35	53±58 [‡]	na	na
Group 3	9±5	21±12	27±12 [‡]	29±20 [‡]	24±20
Energy charge					
Group 1 (controls)	0.9±0.09	0.8±0.2	0.9±0.5	0.9±0.04	0.8±0.08
Group 2	0.7±0.2	0.6±0.2	0.7±0.1	na	na
Group 3	0.8±0.09	0.8±0.1	0.8±0.07	0.8±0.1*	0.7±0.1

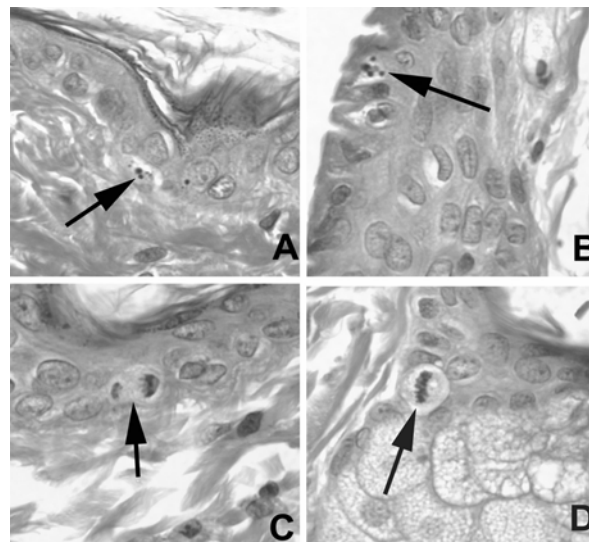
* *P*<0.05, significantly different from controls. † *P*<0.001, significantly different from controls. ‡ *P*<0.05, significantly different from baseline (paired *t* test). § na = not assessed. ww = wet weight. ♦

Figure 1. Apoptosis and mitosis in gut

A and B. Apoptosis identified morphologically (arrows) in the vascularised core of the villus (A) and the crypt (B).
C. An apoptotic cell (arrow) from the villus epithelium has budded into the gut luminal space. These apoptotic cells have morphological features of apoptosis — ranging from condensation and blebbing into apoptotic bodies, to condensation and rounding of the cytoplasm of the apoptotic cell and condensation, budding or margination of the nuclear chromatin — and are found in the gut of hypoxic animals.
D. Mitosis in a section of the gut crypt of a control animal (arrows). (A–D. haematoxylin and eosin stain.)
E and F. Biochemical verification of apoptosis with TUNEL (terminal dideoxyuridine transferase-mediated nick end labelling). Arrows indicate cells with TUNEL-labelled nuclei or nuclear blebs. ◆

with hypoxia in Groups 2 and 3 (Times 2 and 3) and returned to baseline with reoxygenation in Group 3 (Times 4 and 5). Concentrations remained stable in the control group.

Skin lactate: At baseline (Time 1), skin lactate concentrations were comparable between the three groups. At Level 1 and Level 2 hypoxia (Times 2 and 3, respectively), skin lactate concentrations rose significantly in the hypoxia and reoxygenation groups. During reoxygenation, skin lactate concentration continued to remain elevated in the reoxygenation group as compared with controls.

Figure 2. Apoptosis and mitosis in skin

A and B. Apoptosis (arrows) in the epidermis of hypoxic animals. **C and D.** Mitosis in the epidermis (C) and sebaceous gland (D) of control animals. (Haematoxylin and eosin stain.) ◆

Gut lactate: Changes in gut lactate appeared to parallel those of skin lactate. At baseline (Time 1), gut lactate concentrations were comparable between the three groups. With hypoxia, gut lactate concentrations rose significantly in Groups 2 and 3 as compared with Group 1. At Level 2 hypoxia (Time 3), gut lactate concentrations were significantly higher in both the hypoxia and reoxygenation groups compared with the control group. During reoxygenation, gut lactate returned to baseline levels.

Apoptosis scores

Hypoxia was noted to result in apoptosis in the gut villi. In the gut, apoptotic cells were found mainly in the highly vascularised connective tissue core of the villi, in neutrophils as well as connective tissue cells. With respect to gut apoptosis, the scores in the experimental group increased significantly with hypoxia from their baseline values (at Level 2 hypoxia); there was no statistical difference between the three treatment groups at any time point (Table 2). The maximum gut apoptosis score was noted in Group 2 (hypoxia) at Time 3. In the reoxygenation group, some necrosis was found in the peaks of the villi, probably a result of prolonged hypoxia followed by a burst of oxidative stress. At Time 4, apoptosis scores were increased in the controls as compared with baseline.

There was no significant increase in skin apoptosis scores with hypoxia compared with controls ($0.6\% \pm 0.3\%$ in controls, $0.5\% \pm 0.3\%$ in the hypoxia group, and

0.6% ± 1.0% in the reoxygenation group; *P* not significant). In the skin, apoptosis was seen in the epidermis and also in the epithelial cells of hair follicles. Apoptosis of gut and skin is shown in Figure 1 and Figure 2, and immunohistochemical examination demonstrating activation of caspase-3 in hypoxic gut in Figure 3.

Energy charge

Energy charge remained at similar levels at each follow-up time point for each of the three groups (Table 2). However, a statistically significant difference was observed between the experimental group and control group at Time 4.

Relationship between indices of dysoxia (lactate and energy charge) and apoptosis

Spearman's correlation coefficient did not demonstrate a significant correlation between skin lactate concentration and apoptosis ($r=0.21$), gut lactate concentration and apoptosis ($r=-0.28$), or energy charge and apoptosis ($r=0.09$). However energy charge was correlated with gut lactate concentration ($r=0.5$; $P<0.05$).

Discussion

In this experimental model, both control and experimental groups showed evidence of apoptosis, but there was a trend towards increasing apoptosis in the gut villi with progressive severity of tissue hypoxia. Dysoxic changes (evidenced by a decrease in energy charge and an increase in tissue lactate concentration) were noted in conjunction with apoptosis in the experimental arms.

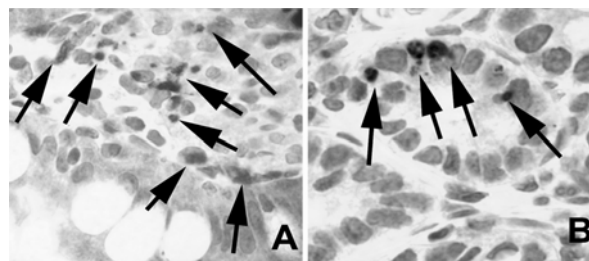
Relationship between dysoxia and apoptosis

As changes in energy charge are more robust markers of dysoxia,²² the positive correlation between changes in tissue lactate concentration and energy charge suggest that the former can be used as a marker of dysoxia. As apoptosis requires the presence of ATP, it begs the question — can dysoxia and apoptosis coexist in the same tissue? Apoptosis is recognised to be energy-dependent. However, it is likely that ATP depletion was not absolute in our model, and the availability of small quantities of ATP was sufficient to maintain the molecular pathways leading to apoptosis.

Comparison with previously published data

A number of animal studies have now established the link between hypoxia and apoptosis (Table 3). Most of these involved incubating cell cultures from animal models under extreme hypoxia or total anoxia, in some cases for prolonged periods, demonstrating varying degrees of apoptosis. Only one published study has examined the relationship between graded hypoxia and apoptosis. Newborn piglets were sub-

Figure 3. Activated caspase-3 in hypoxic gut



Immunohistochemistry was used to identify the presence of cells positive for activated caspase-3 protein (arrows), here using gut from hypoxic animals.

A. The vascularised villus core had multiple positive cells.

B. Gut epithelial cells also had positive label.

In some cases, label was seen in cells with morphological features of apoptosis and, in other cases, in apparently normal cells.

Expression of activated caspase-3 is said to be one of the final components of the apoptotic pathway, pushing the cell into the cellular disaggregation of apoptosis. ♦

jected to varying grades of arterial hypoxaemia induced by F_{iO_2} settings ranging between 0.05 and 0.15 for 1 hour.²³ At the end of the experiment, cerebral cortical tissue was examined for the presence of apoptosis and measurement of nucleotide concentrations. The extent of DNA fragmentation, used as an index of apoptosis, was shown to be proportional to the depletion of tissue energy stores (as measured by nucleotide concentrations). While this was new information, no data were presented on arterial and tissue gas tensions. Hence, critical PO_2 levels for apoptosis could not be determined. Our study is the first to attempt to define the link between clinically relevant PO_2 thresholds and apoptosis in the gut. Diebel et al have reported that exposure of intestinal epithelial cell layers to hypoxia alone was not sufficient to induce apoptosis, and that a superimposed bacterial insult was necessary.²⁴ This finding is at variance with our results. The experimental conditions in our study differed from those of Diebel et al and were more in keeping with what is seen in critical illness, while our results were consistent with data from Hotchkiss et al.⁷

Justification of site, PO_2 thresholds and duration of tissue hypoxia

Subcutaneous PO_2 was chosen because of the strong concordance between subcutaneous and gut luminal gas tensions in our previously described animal models of haemorrhagic and endotoxic shock.^{1,2} As noted above, we chose a threshold tissue PO_2 of 30 mmHg based on our observations in a previous animal study of haemorrhagic shock that the nadir subcutaneous tissue PO_2 was close to 30 mmHg at the height of shock. Furthermore, the normal end-capillary venous oxygen tension, a close approximation

Table 3. Summary of studies that link hypoxia and apoptosis

First author	Model	Tissue	Method	Findings
Yaniv ¹¹	Rodent	Ventricular myocyte	Hypoxia (1% oxygen for 22 h) or normoxia	Increase in apoptosis in the hypoxic group
Ding ⁸	Rodent	Ileal mucosa culture	a) Normoxia (95% O ₂ , 5% CO ₂), b) Normoxia + bacteria c) Anoxia followed by normoxia d) Anoxia + bacteria	Apoptosis in all groups except group (a); highest apoptosis in group (d)
Guo ⁵	Rodent	Ventricular myocyte	Anoxia or normoxia for 16 h	Increase in apoptosis in the hypoxic group
Holleyman ¹⁰	Human	Endothelial cultures	Ischaemia–reperfusion 2 hours of 100% N ₂ followed by 21% O ₂	Marked increase in apoptosis in the ischaemia–reperfusion group
Gill ⁹	Rodent	Brain	Total middle cerebral artery and common carotid artery occlusion, hypoxia (7.7% O ₂)	Apoptosis only in hypoxic group, not in ischaemic group
Pozzi ⁶	Rodent	Langendorf heart preparation	Low-flow normoxia, low-flow hypoxia for 6 hours, no-flow ischaemia for 90 min, followed by reperfusion	Apoptosis similar in all three groups
Akhter ²³	Pig	Brain	Graded hypoxia for 1 hour (5%–15% O ₂) versus normoxia	Severity of apoptosis proportional to severity of hypoxia
Current study	Rodent	Gut and skin	Graded tissue hypoxia (interstitial PO ₂ 0–30 mmHg) versus controls	Increase in apoptosis in the hypoxic limb

of tissue PO₂, is about 37.5 mmHg.¹⁵ Thus, perturbations in oxygen delivery, even of small magnitude, can result in reductions in PO₂ to below 30 mmHg. The 20-minute duration of observation at each level of hypoxia was chosen because of the findings of Hotchkiss et al that apoptosis of the gut mucosa was demonstrable after 20 minutes of shock following trauma.⁷

Implications of the findings

We previously demonstrated concordance in tissue gas tensions in the gastrointestinal tract and skin during shock states. In this study, we demonstrated concomitant histological changes in the gut with tissue hypoxia. The precise implications of intestinal epithelial apoptosis are unclear. Intestinal epithelial apoptosis is known to result in altered epithelial cell permeability and may be one of the initiating pathways for the development of multiple organ dysfunction syndrome.¹² In a human study of 36 critically ill patients (20 sepsis, 16 non-sepsis), there was extensive lymphocytic and intestinal epithelial apoptosis in the group with sepsis compared with the non-sepsis group.²⁵ Gut epithelial apoptosis has been shown to be detrimental to survival in animals with sepsis.¹⁴ Knowledge of critical tissue PO₂ thresholds may provide the clinician with practical resuscitation end-points in hypoxia and shock, and might even modify the practice of “permissive hypoxia” in severe respiratory failure.²⁶

Limitations of the study

Although we demonstrated a significant increase in apoptosis scores with hypoxia in the experimental arms (intragroup),

there was no difference in comparison with controls. Possible explanations for this finding include the small sample size and consequent low power of the study; need for a longer duration of insult to detect identifiable apoptotic changes; and the well-known technical pitfalls associated with identification and quantification of apoptosis.²⁷ Histological analyses reflect only the late phases of the process. Other techniques, such as the TUNEL assay, depend on proteolytic digestion and concentration of the terminal dideoxyuridine transferase enzyme. Immunohistochemistry also identifies only one step of this complex process — caspase-3 activation. Thus, despite a plethora of techniques available to quantify cell death, there is still potential for uncertainty in its recognition. A small increase in apoptosis scores was also noted in the control arm, which was perplexing. Hypoxia and hypoperfusion were not contributory, as arterial and tissue PO₂ and plasma lactate concentration were within the reference range. Harvesting of multiple samples from an animal might lead to pathology. In addition, the removal of serial aliquots of blood may contribute to hypovolaemia. However, the need to obtain sequential information necessitated serial sampling. In addition, MAP was monitored continuously and, apart from brief perturbations in MAP during blood sampling, no significant haemodynamic insult resulted from that sampling. Another possible explanation for apoptosis in the control group could be mechanical ventilation. Experimental data have demonstrated that mechanical ventilation, even of normal lungs, may release pro-apoptotic factors into the systemic circulation, resulting in end-organ apoptosis.²⁸ This might explain the increase in apoptosis scores in the control group.

In conclusion, in this pilot study, reductions in interstitial PO₂ to less than 30 mmHg were associated with significant dysoxic changes in the gut and skin. No clear threshold of tissue PO₂ was identified for the initiation of apoptosis. Further large-scale studies with refinements to the experimental model may allow more precise identification of PO₂ thresholds that are critical for the development of apoptosis.

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