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The Regulation of Interleukin-8 by Hypoxia in Human Macrophages—A Potential Role in the Pathogenesis of the Acute Respiratory Distress Syndrome (ARDS)

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Abstract

Background: The acute respiratory distress syndrome (ARDS) represents a form of severe acute inflammatory lung disease. We have previously demonstrated significantly raised interleukin-8 (IL-8) levels in the lungs of at-risk patients that progress to ARDS, and identified the alveolar macrophage as an important source of this chemokine. We wished to extend this study in a well-defined group of patients with major trauma, and to investigate potential mechanisms for rapid intrapulmonary IL-8 generation.

Materials and Methods: Patients with major trauma underwent bronchoalveolar lavage (BAL) and IL-8 levels were measured in BAL fluid by ELISA. Human macrophages were derived from peripheral blood monocytes from healthy volunteers. Rabbit alveolar macrophages were obtained from *ex-vivo* lavage of healthy rabbit lungs. Macrophages were cultured under normoxic or hypoxic (PO₂ 26 mmHg) conditions. IL-8 and other proinflammatory mediator expression was measured by ELISA, northern blotting or multi-probe RNase protection assay.

Results: In patients with major trauma, IL-8 levels were significantly higher in patients that progressed to ARDS

compared to those that did not (n = 56, P = 0.0001). High IL-8 levels negatively correlated with PaO₂/FiO₂ (r = -0.56, P < 0.001). In human monocyte derived macrophages hypoxia rapidly upregulated IL-8 protein (within 2 hours) and mRNA expression (within 30 mins). Acute hypoxia also increased rabbit alveolar macrophage IL-8 expression. Hypoxia increased DNA binding activity of AP-1 and C/EBP but not NF-κB. Hypoxia induced HIF-1 expression, but cobaltous ions and desferrioxamine did not mimic hypoxic IL-8 induction. Hypoxia downregulated a range of other proinflammatory mediators, including MCP-1 and TNF-α. Both the pattern of cytokine expression and transcription factor activation by hypoxia was different to that seen with endotoxin.

Conclusions: Rapidly raised intrapulmonary IL-8 levels are associated with ARDS progression in patients with major trauma. Acute hypoxia, a clinically relevant stimulus, rapidly and selectively upregulates IL-8 in macrophages associated with a novel pattern of transcription factor activation. Acute hypoxia may represent one of potentially several proinflammatory stimuli responsible for rapid intrapulmonary IL-8 generation in patients at-risk of ARDS.

Introduction

The acute respiratory distress syndrome (ARDS) represents a severe form of acute inflammatory lung disease. The lung injury is characterised by an intense, predominantly neutrophilic, inflammatory infiltrate in the pulmonary vasculature and airspaces (1–3). There is currently no specific therapy for this disorder and the associated mortality is 50% or higher (4). A variety of clinical conditions or insults including major trauma, sepsis syndrome, acid aspiration and severe pneumonia, predispose to the development of ARDS, typically several hours or days after the initial

insult. Hence, ARDS often occurs in the context of a severe systemic inflammatory response. Only a subgroup of between 2–35% of at-risk patients however progress to full-blown ARDS (5).

We have previously observed that in bronchoalveolar lavage (BAL) fluid samples taken within a few hours of the initiating insult, levels of interleukin-8 (IL-8) were significantly higher in those that progressed to ARDS compared to those that did not. Interleukin-8 is a potent neutrophil chemokine, and immunohistochemical analysis has implicated the alveolar macrophage as an important source of this mediator (6). Other investigators have also identified this chemokine as an early marker of inflammation, present in tissue before the onset of neutrophil influx (7,8). Recently, raised intrapulmonary IL-8 levels in donor lungs have been shown to be

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associated with development of graft dysfunction in the recipients (9). Investigating potential mechanisms by which intrapulmonary IL-8 is rapidly generated therefore may be key to our understanding of the pathogenesis of lung injury.

In this paper we report our extended studies of multiple trauma patients, a well-defined group 'at-risk' of developing ARDS. We confirm our earlier finding that raised bronchoalveolar lavage (BAL) IL-8 levels in the very early risk period (median 95 mins post-trauma) are closely associated with ARDS disease progression. The mechanisms by which macrophages may rapidly upregulate IL-8 under such circumstances are unknown. Bacterial endotoxin-derived lipopolysaccharide (LPS) is a classical proinflammatory stimulus that may play a role in the development of the systemic inflammatory response and acute lung injury following major trauma (10,11). However, we found no association between circulating or intrapulmonary endotoxin and IL-8 levels or progression to ARDS. We postulated that physiological events occurring in the first few hours following admission to the Emergency Room may result in increased IL-8 generation. A relationship between patient hypoxia at presentation, as manifest by a reduced PaO₂/FiO₂ ratio, and raised alveolar IL-8 levels was identified, invoking the hypothesis that hypoxia may play an initial role in IL-8 generation.

In *in vitro* studies of human macrophages we have demonstrated that acute hypoxia rapidly and selectively upregulated IL-8 generation. The hypoxic stimulus induced a pattern of chemokine and transcription factor activation significantly different from that seen with LPS. However, IL-8 generation by hypoxia *in vitro* was found to be less than could account for the differences observed in patient studies. This suggests that in addition to hypoxia, other proinflammatory stimuli such as reoxygenation and pulmonary barotrauma during resuscitation may also play a role in rapid intrapulmonary IL-8 production.

Materials and Methods

Patient Studies:

Patient enrolment and Bronchoalveolar Lavage—Consecutive patients with severe multiple trauma and requiring intubation in the Emergency Room (n = 56) were enrolled at the Royal Infirmary Edinburgh, Scotland over a period of 36 months. Consent was obtained from relatives or guardians and the study approved by the Lothian Health Board Ethics Committee. The extent of trauma was assessed by the injury severity score (ISS); severe trauma was defined as an ISS score of ≥ 15 (12). A chest radiograph was taken on all patients as soon as clinically appropriate in the emergency room. The initial ratio between arterial oxygen tension (PaO₂) and fractional concentration of inspired oxygen (FiO₂), a measure of patient oxygenation and gas exchange, was recorded in the emergency room. All patients intubated for clinical indications

underwent bronchoalveolar lavage (BAL) in the emergency room. The technique for BAL and processing of fluid obtained has previously been described (6). A 10 ml venous blood sample, obtained immediately prior to BAL, was centrifuged at 400 g for 10 minutes at 4°C and the plasma stored at -70°C for later analysis. Measurement of BAL fluid IL-8, by standard ELISA as previously described (6). BAL fluid total protein was measured using a commercially available kit (Pierce, Rockford, IL). Serum and BAL endotoxin levels were measured by the limulus amoebocyte lysate assay as previously described (13). Subsequent development of ARDS was defined using the European/American Consensus criteria (13) which requires the presence of bilateral pulmonary infiltrates on the chest radiograph and a PaO₂/FiO₂ ratio of ≤ 200 mmHg.

In Vitro Studies:

Cell Isolation and Culture

Human macrophages—Blood from normal, healthy volunteers was collected in citrated tubes and peripheral blood mononuclear cells (PBMCs) obtained by separation through Percoll (Pharmacia Biotechnologies, St. Albans, UK) density gradient centrifugation as previously described (14,15). Autologous serum was prepared by adding CaCl₂ (10 mM final concentration) to plasma and incubating at 37°C for 1 hour. PBMCs were resuspended in complete medium (Iscove's Modified Dulbecco's Medium supplemented with 1 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, Life Technologies, Paisley, UK) at a concentration of 4×10^6 cells/ml and 3 ml aliquots plated into 35 mm plastic culture wells (CorningCostar, High Wycombe, UK). Monocytes were allowed to selectively adhere for 1 hour in standard conditions (37°C in humidified 95% air/5% CO₂). Non-adherent cells were washed away with warm complete medium and adherent monocytes overlaid with 3 ml of complete medium supplemented with 10% autologous serum. Monocytes were allowed to mature to macrophages over 6 days following which overlying medium was removed and the cells gently rinsed with warm medium.

Rabbit alveolar macrophages—Adult female specific pathogen-free (SPF) New Zealand white rabbits (Charles River Ltd. Margate, Kent) were sedated with intramuscular fentanyl citrate and fluanisone (Hypnorm® 0.3 ml/kg bolus followed by 0.3 ml/kg/h, Janssen-Cilag Ltd., High Wycombe, UK) then anaesthetised with intravenous (i.v.) midazolam (Hypnovel® @ 2 mg/ml, 0.75 ml/kg, Roche Products Ltd., Welwyn Garden City, UK) via marginal ear vein. A tracheotomy was performed and a purpose-made endotracheal tube inserted and secured. Animals were then euthanased with sodium pentobarbitone (Sagatal, 200 mg/ml, Rhone Merieux Ltd., Harlow, Essex) and the lungs immediately removed and placed on ice. Both lungs were lavaged *ex-vivo* by sequential instilling and aspiration of 40 ml aliquots of

cold (4°C) PBS via a quill placed in the tracheostomy tube. Lavaging was continued until there was an appreciable loss of opalescence in the lavage fluid. Alveolar cells were washed in PBS (4°C) twice and resuspended in complete medium. This process yielded alveolar macrophages of >98% purity >95% viability, as assessed by Wright-Giemsa staining of cytopins and trypan blue exclusion (data not shown). Alveolar macrophages were aliquoted in 6-well plates at 4×10^6 cells/3 ml complete medium with 10% heat-inactivated foetal calf serum (FCS) per well. Cells were cultured overnight under standard conditions to allow adherence and then used in hypoxia experiments.

Hypoxic incubation of macrophages—Cells to be cultured under normoxic conditions were placed in a standard incubator at 37°C, 95% air/5% CO₂. In preliminary experiments, cells to be incubated under hypoxic conditions were placed in an anaerobic incubator at 37°C (Napco, Precision Scientific Inc, Ill. USA) with an infused gas mixture of 95% N₂/5% CO₂. We established that our key finding with regard to IL-8 protein secretion and transcription were reproducible in an alternative anaerobic chamber (MK3 Anaerobic Workstation, Don Whitley Scientific Ltd. UK) which maintained an environment at 37°C/80% N₂/10% H₂/10% CO₂. This latter chamber was subsequently used in hypoxia experiments. For both normoxia and hypoxia experiments, cells were overlaid with 1 ml of preincubated complete medium supplemented with 2% autologous serum (or 10% FCS for rabbit alveolar macrophages). The PO₂ of the normoxic and hypoxic medium at steady-state was ~150 mmHg and ~26 mmHg respectively as measured by dissolved oxygen meter (ISO₂ with OXEL-1 probe, World Precision Instruments, Inc. Florida, USA). Cells were cultured for up to 2 hours and viability was not affected as measured by trypan blue exclusion and LDH assay (data not shown).

Supernatants overlying adherent macrophages were collected by gentle aspiration. Adherent macrophages were immediately placed on ice and lysed for protein or RNA extraction. In the case of hypoxic cells, these manipulations were performed inside the anaerobic chamber to avoid reoxygenation.

Enzyme-linked Immunosorbent Assay

Monoclonal and biotinylated anti-human IL-8, tumour necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) antibodies were obtained from R&D Systems and used at concentrations recommended in the manufacturers ELISA protocol. Briefly, flat-bottomed 96-well microtitre plates (E.I.A./R.I.A. plate, Costar, Cambridge, MA) were coated with 100 μ l per well of the monoclonal antibody in PBS overnight at room temperature (RT), washed and non-specific binding sites blocked with 1% BSA in PBS. 100 μ l aliquots of macrophage-derived supernatants or cell lysates (obtained by lysing cells in 0.1% Nonidet-P40 in PBS) were added and incubated for 2 hours at RT.

After washing, 100 μ l per well of secondary biotinylated antibody was added and the plates incubated for 2 hours at RT. Plates were washed and 100 μ l streptavidin-peroxidase conjugate (Dako, Denmark) diluted 1 in 20,000 was incubated in the wells for 20 minutes. Substrate solution consisting of TMB 100 μ g/ml, 4.5 mM H₂O₂ in 0.1 M sodium acetate-citrate pH 4.9 was added and the plates incubated in darkness at RT to the desired extinction. The reaction was stopped with 50 μ l of 0.5 M H₂SO₄. Plates were read at 450 nm in a plate reader (Dynatech MR5000). Sample results were extrapolated from a standard curve derived with recombinant human (rh) IL-8 (WHO International Standard, NIBSC, Herts, UK), rhTNF- α or rhIL-1 β (R&D Systems).

RNA Extraction and Northern Blotting

Total RNA from cells was extracted using TRIzol reagent (Life Technologies) as per the manufacturers protocol. Equal quantities of RNA (15 μ g per lane) were separated on a 1.2% formaldehyde-agarose gel (40 mM 3-morpholinopropanesulphonic acid, pH 7.0, 10 mM sodium acetate, 1 mM EDTA, 2 M formaldehyde) and then transferred to nylon membrane (Du Pont NEN Genescreen Plus) by capillary action in 20 \times SSC. The RNA was wet cross-linked under UV-light and pre-hybridised for 2 hr at 65°C in buffer containing 0.5 M NaHPO₄, 7% SDS and 1 mM EDTA (pH 7.2). Fresh buffer containing (α -³²P) dCTP-labelled IL-8 cDNA probe was then added and hybridisation continued for 18 hours at 65°C. The membranes were washed three times in 2 \times SSC, 0.1% SDS, once at 65°C and twice at room temperature. Membranes were exposed to Biomax MR or X-Omat films (Kodak) with intensifying screens at -70°C. Membranes were subsequently stripped with boiling 0.1% SDS and re-probed with a housekeeping 18s cDNA probe.

Probe Synthesis and Labelling

A 747 bp length human IL-8 cDNA probe was generated by RT-PCR from mRNA derived from human monocytes stimulated with LPS. The primers used were 5'-ATT-TCT-GCA-GCT-CTG-TGT-GA-3' and 5'-TGT-GGA-TCC-TGG-CTA-GCA-GA-3'. Fifty nanograms of IL-8 probe was labelled with 50 μ Ci of (α -³²P) dCTP (Amersham Life Science Ltd., Bucks, UK) as per the manufacturers protocol (ReadyToGo™, Pharmacia Biotechnologies, St. Albans, UK). Twenty nanograms of the 18s housekeeping cDNA probe (a generous gift from Dr Irfan Rahman, Edinburgh, UK) was similarly labelled.

Rabbit Interleukin-8 Reverse Transcription PCR (RT-PCR)

IL-8 mRNA expression from rabbit alveolar macrophages was determined by semi-quantitative RT-PCR. First strand cDNA was synthesised from 1 μ g total RNA using AMV reverse transcriptase (Promega, Southampton, UK). Rabbit IL-8 primers, designed from the GenBank succession sequence number

M57439 (16) were; 5'-ATG AAC TCC AAG CTG GCT-3' (sense) and 5'-TTA TGA CTC TTG CTG CTC AGC-3' (antisense). PCR conditions were: 94°C for 1 min (denaturation), 55°C for 1 min (annealing) and 72°C for 1 min (extension) for 26 cycles followed by a final 5 min extension at 72°C. IL-8 product was expressed relative to that obtained by RT-PCR for the house-keeping gene human β -actin, the primers for which were: 5'-CCA CCA ACT GGG ACG ACA TG-3' (sense) and 5'-GTC TCA AAC ATG ATC TGG GTC ATC-3' (antisense). The PCR conditions for β -actin were: 94°C for 1 min (denaturation), 60°C for 1 min (annealing) and 72°C for 1 min (extension) for 26 cycles followed by a final 5 min extension at 72°C. Preliminary experiments had established that a limited cycle number of 26 yielded PCR product within the linear amplification range for both IL-8 (306 base pairs) and human β -actin (180 base pairs).

Electromobility Gel-Shift Assay

Total nuclear proteins were extracted from cells using a method modified from that previously described (16). Briefly, macrophages were kept on ice and scraped into cold buffer containing 10 mM HEPES—KOH pH 7.8, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA protease inhibitor cocktail (Complete™, Boehringer Mannheim). The cells were allowed to swell on ice for 15 mins then lysed by adding 0.4% Nonidet P40 and centrifuged (12000 *g* for 1 min at 4°C). The supernatant was carefully removed and the pellet resuspended in 50 μ l of cold buffer containing 50 mM HEPES—KOH pH 7.8, 50 mM KCl, 300 mM NaCl, 1 mM dithiothreitol, 10% glycerol, 0.1 mM EDTA and protease inhibitor cocktail. Following 20 mins incubation at 4°C with gentle agitation and centrifugation (12000 *g* for 1 min at 4°C) the supernatant was carefully removed and protein content assayed by BCA protein assay (Pierce, Rockford, IL). The binding reaction was performed at RT as follows: 4 μ g aliquots of protein were incubated for 5 mins in binding buffer containing 5 mM Tris, 0.5 mM EDTA, 8% glycerol, 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM DTT and 1.2 μ g of polydI-dC and then a further 15 mins with an appropriate (γ -³²P)-labelled oligonucleotide. In the case of supershift assays nuclear extracts were first co-incubated at 4°C with rabbit anti-human polyclonal IgG antibodies to p50 and p65 (Santa Cruz) for 4 hours or with antibodies to c-jun and c-fos (Santa Cruz) for 20 hours in binding buffer minus DTT. These were subsequently incubated at RT with appropriate (γ -³²P)-labelled oligonucleotide in the presence of 0.5 mM DTT. The reaction mixtures were separated on a 6% polyacrylamide gel at 180 V at room temperature in 0.5% TBE buffer. After drying, gels were exposed to X-ray film and densitometry performed. The double stranded oligonucleotide probes used were as follows: AP-1 5'-AGT GTG ATG ACT CAG GTT TGC-3', C/EBP 5'-CAT CAG TTG CAA ATC GTT AAC-3', NFkB 5'-TCG TGG AAT TTC CTC TGA CAT-3'.

Immunoblotting for HIF-1 α Protein in Macrophages

Total cellular protein was extracted by a Trizol base method as per the manufacturers protocol. Immunoblotting for HIF-1 α with MoAb 28B has been previously described (17). Briefly, proteins were resolved in SDS/10% polyacrylamide gel, transferred overnight to Immobolin P (Millipore, Bedford, MA) and exposed to HIF-1 α antibody. Detection was with HRP-conjugated goat anti-mouse Igs (DAKO, Ely, UK) and enhanced chemiluminescence. After analysis, membranes were stained with Ponceau S to verify equal protein loading and transfer.

RNAse Protection Assay for Multiple Chemokines

RNAse protection assays were performed using the RiboQuant® multi-probe RNAse protection assay system (Pharmingen, San Diego, CA) as per the manufacturers protocol. Briefly, total RNA from human macrophages was obtained as described. (α -³²P)-labelled anti-sense RNA probes were generated from multi-probes using T7 polymerase. Multi-probe hCK-5 contains multiple chemokine templates. Multi-probe hCK-3 includes a TNF- α template. Both probe sets include templates for the house-keeping genes L-32 and GAPDH. Five micrograms of total RNA was hybridised with 3×10^5 cpm of labelled hCK-5 or hCK-3 probe at 56°C overnight. Free-probe and other single stranded RNA was digested with RNAse A and protected hybridised RNA species were resolved on a 6% denaturing polyacrylamide sequencing gel and exposed to X-ray film at -70°C.

Statistical Analysis

Patient data is presented as mean \pm SD and analysed by the non-parametric Mann-Whitney U test. Correlation was analysed by Pearson *r*. *In vitro* data is presented as mean \pm S.E.M unless otherwise stated and analysed by Mann-Whitney U or *t*-test for normally distributed data. Significance testing was two-tailed and a value of $P < 0.05$ was considered significant.

Results

Patient Studies

Fifty-six trauma patients, median age 48 years (range 18–89) were enrolled in the study. The median ISS was 25 (range 16–66). A chest radiograph was taken and reviewed within 4 hours of admission for all patients. No patients had radiographic evidence of ARDS at this time. BAL sampling as performed at a median time of 95 mins (range 30–240) after the trauma event. Seventeen patients subsequently progressed to ARDS and mean BAL IL-8 (pg/ml) levels were significantly higher in these patients compared to those who did not (1425 ± 1841 v 206 ± 288 pg/ml, $P = 0.0001$) (Fig. 1). Bronchoalveolar lavage total protein levels (as a measure of lung leak), endotoxin levels, and neutrophil counts were not significantly different between patients who did and did not progress to ARDS (Table 1.) and in 7 of the

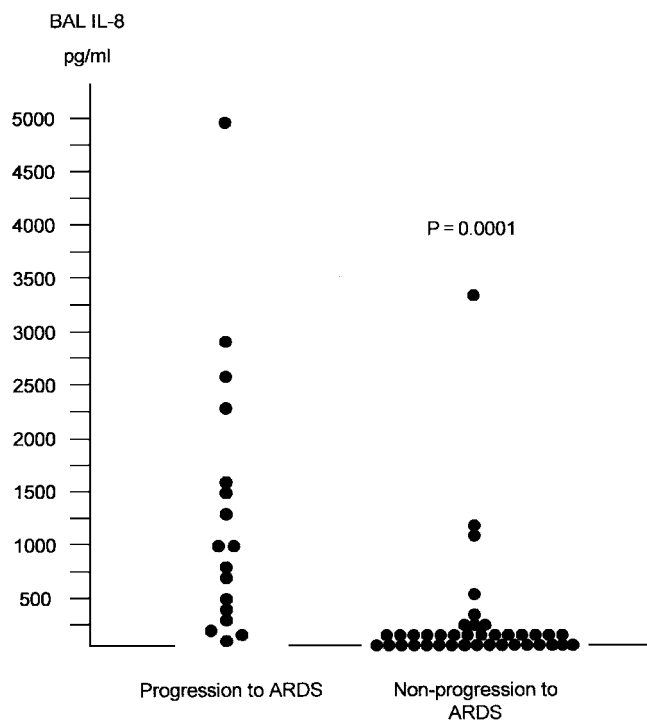


Fig. 1. Bronchoalveolar lavage (BAL) levels of IL-8 in patients that did and did not progress to ARDS. BAL IL-8 levels were significantly higher in those patients that progressed to ARDS (n = 56, P = 0.0001).

17 patients who progressed to ARDS, BAL endotoxin levels were at the lower limit of detection (2.5 pg/ml). Plasma endotoxin levels in patients who did and did not progress to ARDS were also not significantly different (8.2 ± 23 v 6.7 ± 35 pg/ml, P = 0.65).

A reliable initial PaO₂/FiO₂ ratio was available in n = 41 patients. The median value was 315 mmHg (range 204–460). Hence, the majority of trauma patients had evidence of significantly impaired gas exchange at the time of recruitment. A correlation was observed between degree of hypoxia as manifested by reduced PaO₂/FiO₂ ratio and raised BAL IL-8 levels ($r = -0.56$, P < 0.001) (Fig. 2). The influence of acute hypoxia upon IL-8 generation from human macrophages was investigated.

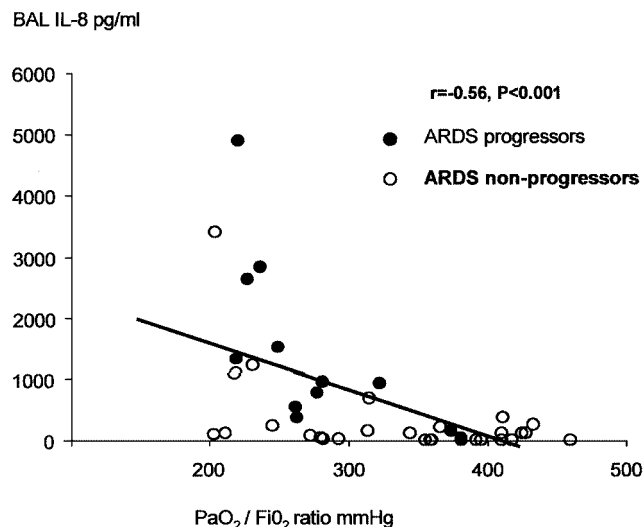


Fig. 2. Correlation between PaO₂/FiO₂ ratio and raised IL-8 at admission. Initial PaO₂/FiO₂ ratio plotted against BAL IL-8 for trauma patients (n = 41). A negative correlation was observed between reduced PaO₂/FiO₂ ratio and raised BAL IL-8 ($r = -0.56$, P < 0.001).

In Vitro Studies

IL-8 Secretion from Hypoxic Human Macrophages

Hypoxia was found to double IL-8 protein secretion from macrophages by 2 hours compared to normoxic controls (0.94 ± 0.28 v 0.54 ± 0.24 iu/ml (P < 0.02). This rapid increase was similar to that induced with IL-1 β (0.89 ± 0.21) and TNF- α (1.1 ± 0.3) but significantly less than that with LPS (3.8 ± 0.35) (Fig. 3). In order to determine if these findings were, in part, due to release of intracellular stores we measured IL-8 in cell lysates. IL-8 levels in both hypoxic and normoxic cells were below the level of detectability (<4 iu/ml) at both time-points demonstrating the absence of stored IL-8 in these cells. We addressed the possibility that the increase in IL-8 release by 2 hours was secondary to increased TNF- α or IL-1 β synthesis. Supernatant and cell-lysate levels for both cytokines were found to be below the level of detectability (<15.6 pg/ml) and <3.9 pg/ml respectively) by ELISA.

Table 1. Analysis of bronchoalveolar lavage fluid of multiple trauma patients (n = 56) who did and did not progress to ARDS

Bal Fluid	Patients who did not Progress to ARDS	Patients who Progressed to ARDS	P Value
Total protein μ g/ml	190 \pm 98	240 \pm 142	0.6
IL-8 pg/ml	206 \pm 288	1425 \pm 1841	0.0001
Endotoxin pg/ml	4.5 \pm 3.6	5.5 \pm 3.8	0.8
Neutrophils (%)	5 (0.5–10)*	4.5 (1–8)*	0.8

*Results expressed as mean \pm s.d. except [median (range)].

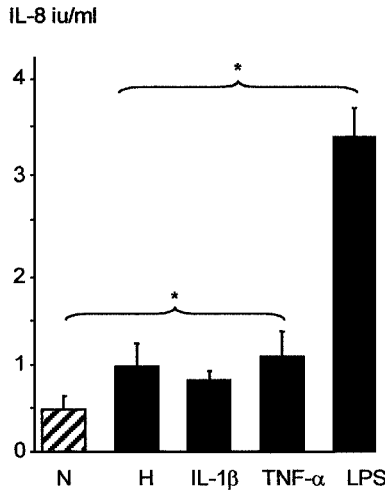


Fig. 3. Hypoxia stimulates IL-8 protein secretion. Human macrophages cultured for 2 hours secreted significantly greater IL-8 than normoxic controls ($n = 9$, $P < 0.02$). The increase was similar to that seen with 2 hours incubation with IL-1 β (10 ng/ml) and TNF- α (10 ng/ml), but significantly less than that induced by LPS (1 μ g/ml) ($n > 4$, $P < 0.01$).

Hypoxia Increases IL-8 mRNA in Human Monocyte-derived Macrophages and Rabbit Alveolar Macrophages

The increase in IL-8 protein secretion at 2 hours prompted us to investigate the influence of short periods of hypoxia upon IL-8 mRNA levels. We found that in human macrophages hypoxia increased steady-state IL-8 mRNA levels at 30 mins, 1 hour, 2 hours (by $90 \pm 50\%$, $104 \pm 38\%$ and $157 \pm 41\%$ respectively, expressed as a percentage increase over normoxic controls) (Fig. 4). Using semi-quantitative RT-PCR, acute hypoxia was found

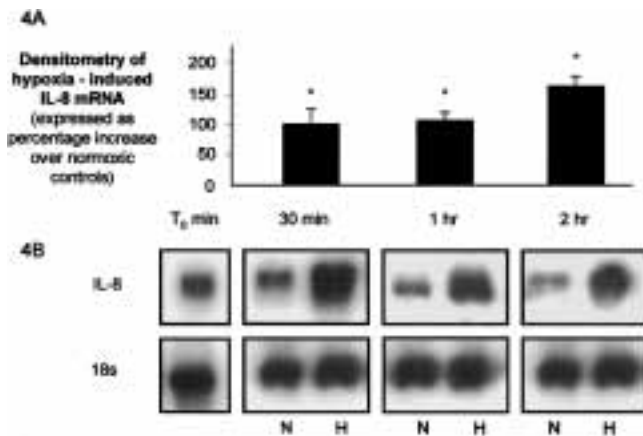


Fig. 4. Hypoxia increases IL-8 mRNA expression in human monocyte-derived macrophages. Human macrophages were exposed to normoxia (N) or hypoxia (H). IL-8 mRNA expression was determined by northern blot analysis. Hypoxia significantly increased IL-8 mRNA as measured by densitometry, expressed as a percentage increase over normoxic controls (A). ($P < 0.01$ for all time points, minimum of $n = 3$). Representative blots are shown (B) and comparable loading is demonstrated with an 18s housekeeping probe.

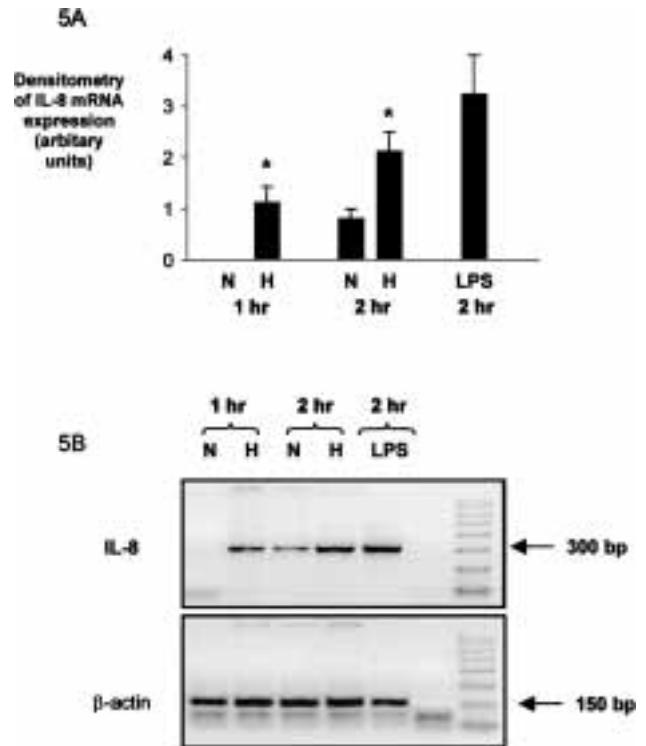


Fig. 5. Hypoxia increases IL-8 mRNA expression in rabbit alveolar macrophages. Rabbit alveolar macrophages were exposed to normoxia (N), hypoxia (H) or LPS. Hypoxia significantly increased steady-state IL-8 mRNA expression compared to normoxic controls by 1 and 2 hours (A). A representative RT-PCR gel is shown (B).

to increase steady-state IL-8 mRNA expression in rabbit alveolar macrophages (Fig. 5).

Transcription Factor Activation in Hypoxic Macrophages

The increase in steady-state IL-8 mRNA might have occurred as a consequence of increased mRNA transcription, stability or both. Our finding that in human macrophages hypoxia increases mRNA levels as rapidly as 30 mins post-exposure suggested that an increase in transcription at least played a part in increasing IL-8 mRNA levels. We therefore studied the effect of hypoxia on transcription factors known to be implicated in the regulation of IL-8 synthesis in human cells.

Since hypoxia induced a rapid upregulation of IL-8 mRNA levels, we first measured by EMSA the nuclear levels of NF- κ B, AP-1 and C/EBP. These transcription factors are known to be important in the regulation of IL-8. We compared the pattern of expression observed with that induced by LPS, a known potent activator of macrophages and stimulus for IL-8 release.

Hypoxia increased nuclear AP-1 levels by $67 \pm 11\%$ and $130 \pm 34\%$ at 15 and 30 mins respectively as measured by densitometry ($P < 0.02$, $n = 4$). (Fig. 6A and 6B). By contrast LPS (1 μ g/ml) did not significantly activate AP-1 by 30 mins of exposure.

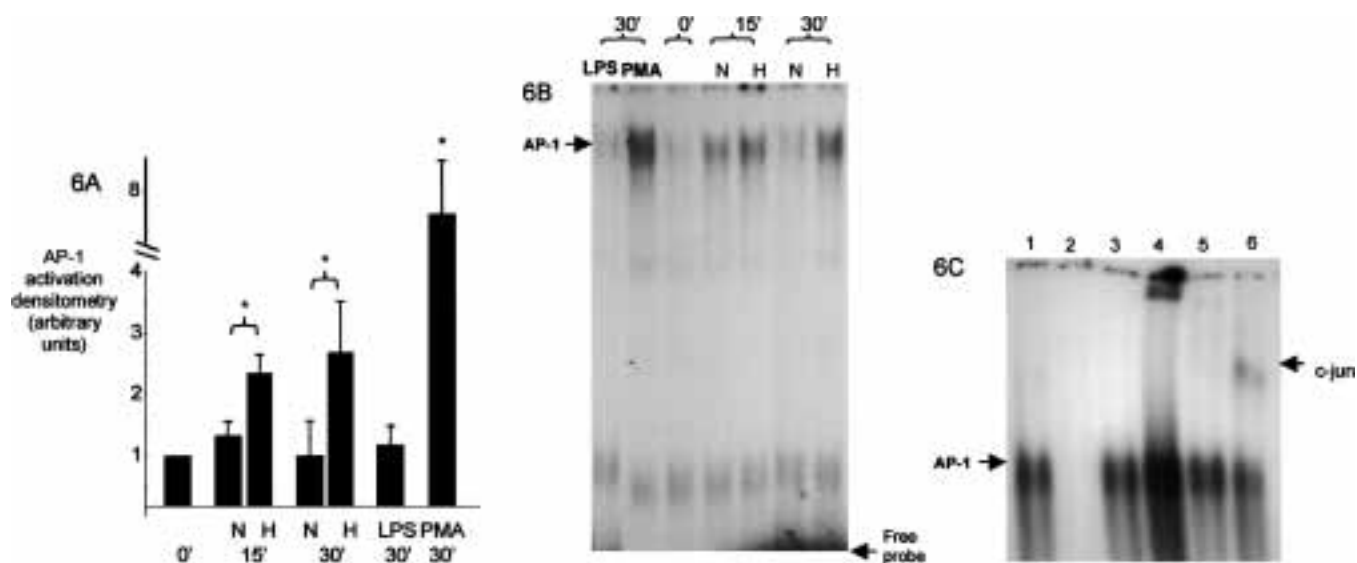


Fig. 6. Hypoxia activates AP-1. Macrophages were incubated under normoxic (N) or hypoxic (H) conditions for up to 30 min. and nuclear levels of AP-1 determined by electromobility gel-shift assay (EMSA). Compared to normoxic controls, (6A) hypoxia significantly increased AP-1 activity at 15 and 30 min. respectively as measured by densitometry (A) ($n = 4$, $P = 0.02$). LPS ($1 \mu\text{g}/\text{ml}$) for 30 min. does not induce AP-1 activation. PMA (100 nM), a known potent upregulator of AP-1 is used as a positive control. A representative EMSA is shown (6B). The specificity and nature of the observed AP-1 band is addressed by super-shift assay (6C). Lane 1 contains $4 \mu\text{g}$ of nuclear protein from hypoxic macrophages incubated with end-labelled AP-1 oligonucleotide probe only. Subsequent lanes are as Lane 1 with the addition of $100\times$ excess cold AP-1 competitor (Lane 2), $100\times$ excess cold non-competitor (Lane 3), $2 \mu\text{l}$ rabbit serum (Lane 4 with non-specific band), c-fos antibody (Lane 5) or c-jun antibody (Lane 6 with super-shifted band).

Phorbol myristate acetate (PMA) (100 nM), a known stimulus for AP-1, was used as a positive control in these experiments. We confirmed the specificity of the AP-1 band by competition assay and demonstrated that the DNA-protein complex induced by hypoxia contained the c-jun member of the AP-1 family but not c-fos. (Fig. 6C)

Hypoxia also increased nuclear C/EBP levels by $79 \pm 9\%$ and $82 \pm 18\%$ at 15 and 30 mins respectively as measured by densitometry (Fig. 7A and 7B). By comparison, LPS induced a $160 \pm 26\%$ increase in levels compared to normoxic controls ($P < 0.02$, $n = 4$). The specificity of the band-shift observed was confirmed by competition assay. (Fig. 7C).

We found that hypoxia for up to 30 mins did not increase nuclear NF- κ B above normoxic levels, demonstrating that hypoxia does not lead to a general increase in transcription factor activation. LPS by contrast increased NF- κ B levels by $322 \pm 34\%$ by 30 mins (Fig. 8A). We confirmed the specificity of this band with a competition study, and supershift assay with LPS-treated macrophages revealed two supershifted bands with a p50 antibody and one with a p65 antibody, suggesting the activated NF- κ B complex contained both subunits. (Fig. 8B).

To further explore a potential activation of NF- κ B by hypoxia we repeated this experiment using a consensus oligonucleotide probe (Promega—see methods), designed to bind to a broader spectrum of NF- κ B subunits. This again revealed increased nuclear NF- κ B levels with LPS ($430 \pm 54\%$ increase

compared to normoxic controls) but no significant effect with hypoxia (Fig. 9)

Role of HIF-1 α in Hypoxia-induced IL-8 Generation

Whilst AP-1, C/EBP and NF- κ B have all previously been implicated in IL-8 gene transcription, the role of HIF-1 α is not known. The regulation of HIF-1 α is predominantly governed by oxygen tension (18). However cobaltous ions and iron chelators mimic hypoxia in both induction of HIF-1 α protein and activation of HIF-1 α -mediated target genes (19–22).

Macrophages exposed to normoxia, hypoxia, CoCl ($100 \mu\text{M}$), desferrioxamine (DFO) (1 mM) or LPS ($1 \mu\text{g}/\text{ml}$) for 2 hours. Immunoblotting for HIF-1 α revealed expression in hypoxic cells and those treated with CoCl and DFO, with no expression in normoxic cells or those treated with LPS (Fig. 10A). However neither CoCl nor DFO increased IL-8 mRNA expression at this time point (Fig. 10B), suggesting HIF-1 α is unlikely to be implicated in the hypoxic induction of IL-8.

Hypoxia Specifically Upregulates IL-8 mRNA

Our clinical data had specifically associated with ARDS progression with raised IL-8 levels rather than a range of other chemokines. We studied the effect of 2 hours hypoxia on steady state mRNA levels of a range of chemokines using a multiprobe RNase protection assay (see methods). In contrast to the increase in IL-8 mRNA observed ($130 \pm 18\%$, expressed as a

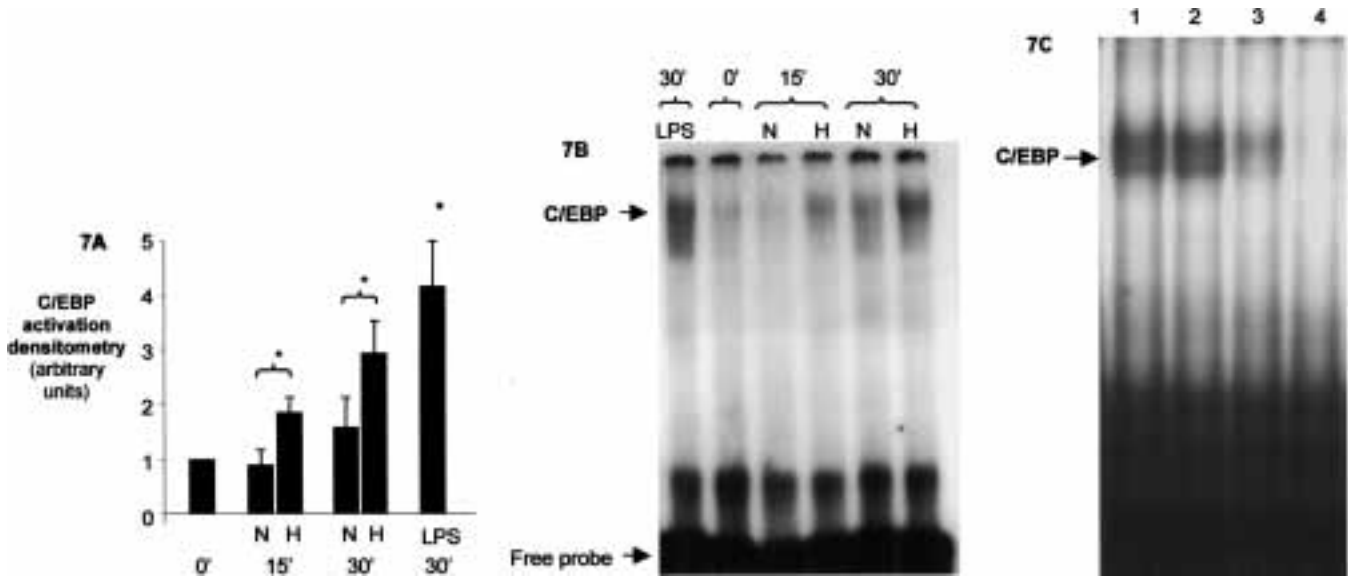


Fig. 7. Hypoxia activates C/EBP. Macrophages were incubated under normoxic (N) or hypoxic (H) conditions for up to 30 min. and nuclear levels of C/EBP determined by EMSA (7A). Hypoxia significantly increased C/EBP activity at 15 and 30 min. respectively as measured by densitometry. LPS (1 $\mu\text{g}/\text{ml}$ for 30 min.) induces a $160 \pm 26\%$ increase compared to normoxic controls ($P < 0.02$, $n = 4$). A representative EMSA is shown (7B). (7C) The specificity of the observed C/EBP band is addressed by competition assay. Lane 1 contains 4 μg of protein from hypoxic macrophages incubated with end-labelled C/EBP oligonucleotide probe only. Lane 2 as Lane 1 with the addition of 100 \times excess cold non-competitor. Lanes 3, 100 \times excess cold C/EBP competitor. Lane 4, 1000 \times excess cold C/EBP competitor.

percent increase over normoxia. $P < 0.01$) the other chemokines assayed were all significantly downregulated by 2 hours hypoxia: RANTES ($62 \pm 12\%$), IP-10 (100%), MIP-1 β ($76 \pm 9\%$), MIP-1 α ($52 \pm 10\%$) and MCP-1 (100%), expressed as percentage inhibition of normoxic controls (all $P < 0.02$). LPS was able to rapidly upregulate RANTES ($590 \pm 94\%$), IP-10 ($60 \pm 38\%$), MIP-1 β ($480 \pm 48\%$), MIP-1 α ($396 \pm 47\%$) and IL-8 ($410 \pm 60\%$) (all $P < 0.02$) (Fig. 11). Hypoxia also had a small but significant inhibitory effect on TNF- α mRNA expression ($38 \pm 8\%$) whilst LPS significantly increased expression

($610 \pm 98\%$) ($P < 0.05$ and $P < 0.02$ respectively). (Fig. 12)

Discussion

In this study we confirmed that in multiple trauma patients at risk of ARDS, raised initial BAL levels of IL-8 were significantly associated with subsequent progression to ARDS. We hypothesised that physiological events in the first few hours following major trauma generated intrapulmonary IL-8 production. We found a significant correlation

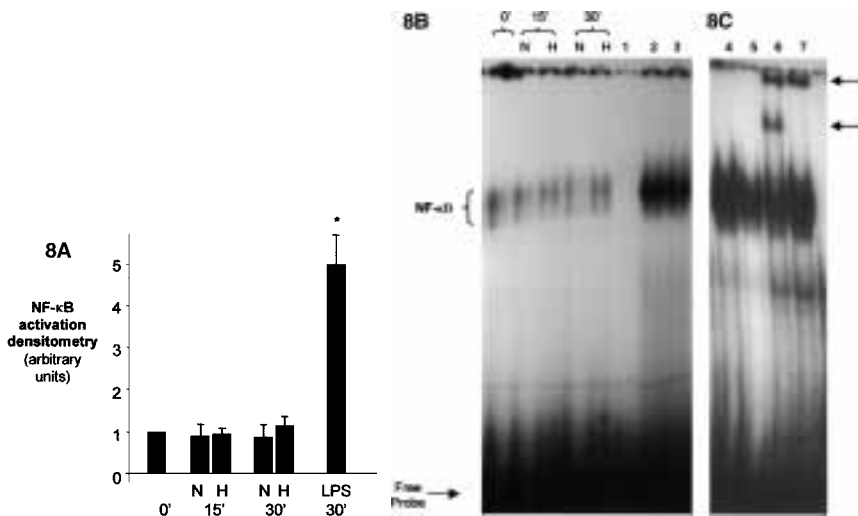


Fig. 8. Hypoxia does not activate NF- κ B. Macrophages were incubated under normoxic (N) or hypoxic (H) conditions for up to 30 min. and nuclear levels of NF- κ B determined by EMSA (8A). Hypoxia does not activate NF- κ B activity above basal normoxic levels. LPS (1 $\mu\text{g}/\text{ml}$ for 30 min.) strongly induces NF- κ B activation ($n = 4$, $P < 0.01$). A representative EMSA is shown including a competition assay (8B). Lane 3 contains 4 μg of protein from LPS-stimulated macrophages. Lane 2 as 3 with the addition of 100 \times excess cold non-competitor. Lane 1, 100 \times excess cold NF- κ B competitor. A supershift assay further defines this LPS-induced band (8C). Lane 4, nuclear extracts from LPS-stimulated macrophages (as Lane 1). Subsequent lanes with addition of 2 μl control rabbit serum (Lane 5), p50 antibody (Lane 6) revealing two distinct supershifted bands) or p65 antibody (Lane 7 revealing one supershifted band).

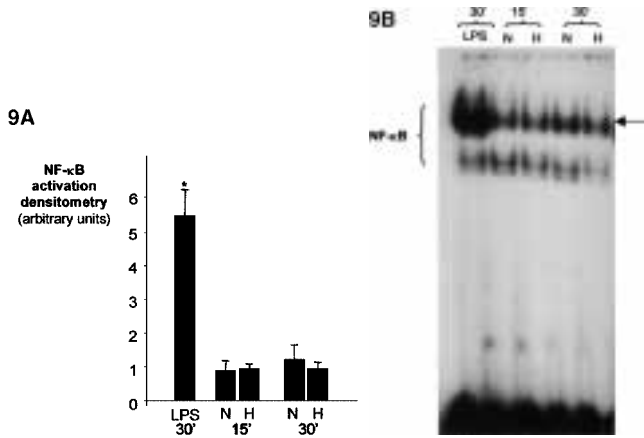


Fig. 9. Hypoxia does not activate NF-κB—using a consensus oligonucleotide probe. Macrophages incubated as previously for 30 min. and nuclear levels of NF-κB determined by EMSA using a consensus oligonucleotide probe (see methods) (9A). Hypoxia does not activate NF-κB activity above basal normoxic levels. LPS (1 μg/ml for 30 mins) strongly induces NF-κB activation. (n = 4, P < 0.01). A representative EMSA is shown, demonstrating that the consensus probe reveals two distinct NF-κB bands, of which the upper (arrowed) is inducible by LPS (9B).

between patient hypoxia, as indicated by a reduced PaO₂/FiO₂ ratio on admission, and raised intrapulmonary IL-8 levels (r = -0.56, P < 0.02). In subsequent *in vitro* studies we demonstrated for the first time that clinically relevant short periods of hypoxia upregulated synthesis of IL-8. Hypoxia was shown to increase DNA binding activity of the transcription factors AP-1 and C/EBP, but not NF-κB, within 15 mins of hypoxic exposure, a pattern of activation which differs from that observed with LPS. Hypoxic macrophages expressed HIF-1α, but our studies with cobaltous ions and DFO suggest that HIF-1α may not be implicated in IL-8 regulation. Both the pattern of transcription factor activation and chemokine expression were different to that observed with the classical proinflammatory mediator, endotoxin.

It is acknowledged that ARDS represents the severe end of a spectrum of acute lung injury (23).

One strategy for investigating the pathogenesis of ARDS is to focus on patients at-risk of developing ARDS, in particular delineating those key *early* inflammatory events which may be associated with subsequent disease progression. Our initial report, based on patients (n = 29) with a variety of risk-factors for ARDS, revealed an association between raised bronchoalveolar lavage (BAL) levels of IL-8 and progression to ARDS (6). In this extended study we have focused on a larger, well-defined at-risk population of trauma victims. Our original observation that raised initial alveolar IL-8 levels are associated with ARDS progression was confirmed. Investigating potential mechanisms by which intrapulmonary IL-8 is rapidly generated may therefore be key to our understanding of the pathogenesis of lung injury.

Several studies have identified raised IL-8 levels in the BAL fluid of patients with established severe lung injury and ARDS (24–26). We are confident that the raised intrapulmonary IL-8 levels detected in our patients was present before the onset of significant lung injury based upon the following observations: 1) we were able to target patients very soon after the trauma event (median 95 min), with no patient recruited after 4 hours. At this time no patient studied had characteristic chest radiographic appearances of ARDS; 2) lung leak, as assessed by total protein concentrations in BAL fluid, was low (240 ± 142 μg/ml in the patients who progressed to ARDS). Previous studies have shown that BAL levels of total protein in established ARDS are 5–10 fold higher than that found in our at-risk population. (27–29); 3) the median BAL neutrophil count was 5% for our at-risk population. Again, this is strikingly lower than would be found in established disease, in which neutrophils typically represent over 50% of the BAL cell population (27,28,30).

Our clinical studies demonstrate a temporal association between IL-8 generation and development of lung injury, though not necessarily causation. However interleukin-8 is regarded as the most potent neutrophil chemokine in the lung (31). In patients with established ARDS, IL-8 has been shown to

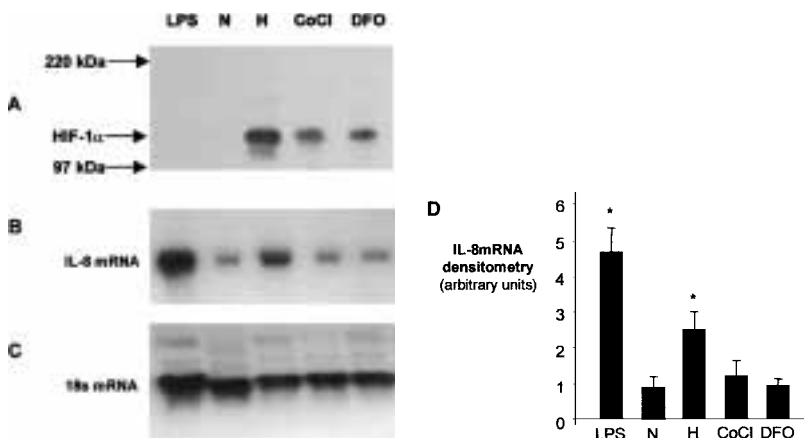


Fig. 10. HIF-1α expression in human macrophages. Macrophages were incubated under normoxic (N) or hypoxic (H) conditions, or treated with LPS, 100 μM CoCl or 1 mM desferrioxamine (DFO), for 2 hours. HIF-1α protein expression was determined by immunoblotting total nuclear extract. Hypoxia, CoCl and DFO all induce HIF-1α, with no expression under normoxic conditions or following LPS treatment (10A). IL-8 mRNA expression was determined by immunoblotting (10B). An 18s housekeeping probe was used to verify equal RNA loading (10C). Relative densitometry, corrected for RNA loading, from n = 3 experiments is shown in panel (10D). Neither CoCl nor DFO mimicked the hypoxia-induced increase in IL-8 mRNA expression.

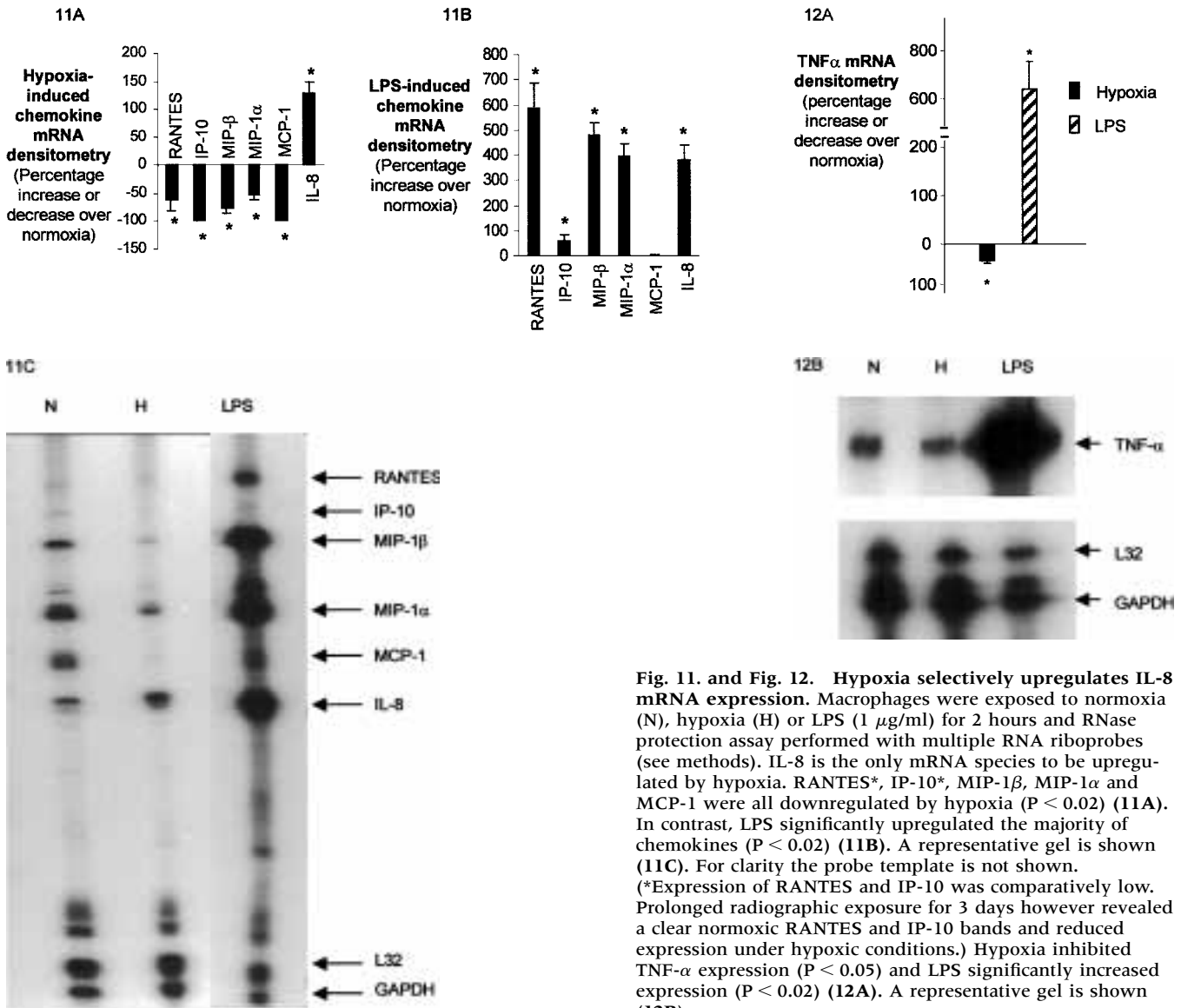


Fig. 11. and Fig. 12. Hypoxia selectively upregulates IL-8 mRNA expression. Macrophages were exposed to normoxia (N), hypoxia (H) or LPS (1 μ g/ml) for 2 hours and RNase protection assay performed with multiple RNA riboprobes (see methods). IL-8 is the only mRNA species to be upregulated by hypoxia. RANTES*, IP-10*, MIP-1 β , MIP-1 α and MCP-1 were all downregulated by hypoxia ($P < 0.02$) (11A). In contrast, LPS significantly upregulated the majority of chemokines ($P < 0.02$) (11B). A representative gel is shown (11C). For clarity the probe template is not shown. (*Expression of RANTES and IP-10 was comparatively low. Prolonged radiographic exposure for 3 days however revealed a clear normoxic RANTES and IP-10 bands and reduced expression under hypoxic conditions.) Hypoxia inhibited TNF- α expression ($P < 0.05$) and LPS significantly increased expression ($P < 0.02$) (12A). A representative gel is shown (12B).

be responsible for over 50% of the neutrophil chemotactic activity of BAL fluid (32). In animal models, acute lung injury secondary to ischaemia/reperfusion (33), alveolar collapse/re-expansion (34) and acid instillation (35) is significantly attenuated by administration of anti-IL-8 antibody. These studies suggest that raised IL-8 levels may therefore play a central role in driving the pulmonary inflammatory response in acute lung injury.

In our *in vitro* studies, monocyte-derived macrophages were exposed to a PO₂ of 26 mmHg. The precursor of the alveolar macrophages is the peripheral circulating monocyte (36–38), hence macrophages matured from monocytes provide a reasonable model for *in vitro* studies. We showed that our principle finding of hypoxic upregulation of IL-8 mRNA was also seen in rabbit alveolar macrophages. We believe that the degree of hypoxia employed may be physiologically

relevant in the alveolar air-spaces of patients with multiple trauma. In the immediate aftermath of major trauma, impaired consciousness, lung atelectasis, pulmonary contusion and intrapulmonary shunting are all likely to contribute to significant alveolar hypoxia and impaired gas exchange (39–42). These factors would account for the reduced PaO₂/FiO₂ observed in our trauma patients in the absence of severe lung injury. Direct measurement of tissue oxygenation *in vivo* was however not feasible in our studies.

We demonstrated that in human macrophages IL-8 protein secretion was increased within 2 hours, and mRNA expression within 30 minutes of hypoxic exposure; a time-frame relevant to our clinical findings. Importantly the degree of stimulation at this time point was similar to that seen with the archetypal early pro-inflammatory mediators TNF- α and IL-1 β . The up-regulation of IL-8 mRNA in macrophages by hypoxia

was rapid, occurring within 30 minutes of exposure. Hypoxia has been reported to increase IL-8 expression in other cell types including endothelial cells (43), fibroblasts and vascular smooth muscle cells (44), although significant protein or mRNA expression did not occur until at least 4 hours hypoxic exposure.

The 5'-regulatory region of the IL-8 gene contains several potential transcription regulatory elements. Three transcription factors in particular, NF- κ B, C/EBP and AP-1 have been implicated in regulating IL-8 gene transcription. The relative importance of these transcription factors appears to be dependent upon both the cell type and the stimulus. Although the NF- κ B binding site is often considered to be the most important (45–47), recent studies provide evidence for NF- κ B independent, AP-1 dependent IL-8 regulation (48,49). In this study we demonstrated that hypoxia rapidly activated both AP-1 and C/EBP, significantly increasing nuclear levels within 15 minutes of exposure compared to normoxic controls. In contrast, there was no hypoxia-induced NF- κ B activation above normoxic levels at the early time-points investigated. This pattern of activation differs from that seen with LPS, which strongly induced NF- κ B activation in particular.

The rapid activation of AP-1 by hypoxia has previously been demonstrated in other cell types (50–52). C/EBP activation by hypoxia has also previously been reported in cultured endothelial cells (53) and in an *in-vivo* model (54). In contrast to AP-1 and C/EBP, we found that human macrophages exposed to up to 30 mins hypoxia did not exhibit nuclear NF- κ B levels raised above normoxic controls. At least two previous studies have implicated NF- κ B in the hypoxic upregulation of IL-8 (43,55). These studies were in different cell lines (human endothelial and bovine retinal glial cells respectively) and employed hypoxia for up to 24 hours. Acute hypoxia has recently been shown to activate NF- κ B in a murine macrophage cell line (56). Our findings do not imply that NF- κ B is superfluous to hypoxia-induced IL-8 transcription. Our experiments revealed basal constitutive activation of all three transcription factors in normoxic human macrophages. It is possible that constitutive activation of NF- κ B is necessary for subsequent hypoxic upregulation. However it is suggested that since both AP-1 and C/EBP are rapidly activated by hypoxia they may have a direct role in the early hypoxic upregulation of IL-8 in human macrophages.

Whilst AP-1, C/EBP and NF- κ B have all been previously implicated in IL-8 gene transcription, the role of HIF-1 α is not known. A computer-assisted search (TFSEARCH version 1.3, Yutaki Akiyama, Kyoto University, Japan) for the core HIF-1 binding sequence 5'-CGTG-3' in the published 1.5 Kb IL-8 promoter region (Accession no. M28130) (57) did not reveal any putative HIF-1 binding sites 5' to the TATA box. However, HIF-1 is considered a central regulator of a hypoxia-mediated gene transcription (58) and HIF-1 binding elements may be distant to the transcription

initiation site. The regulation of HIF-1 α is predominantly governed by oxygen tension (18). However cobaltous ions and iron chelators mimic hypoxia in both induction of HIF-1 α protein and activation of a number of HIF-1 α -mediated target genes (19–22). Our observation that HIF-1 α expression was induced by hypoxia, cobaltous ions and DFO, but that these pharmacological mimics of hypoxia did not increase IL-8 mRNA expression suggests that HIF-1 α may not be involved in IL-8 regulation.

Finally, we considered the potential selectivity of the IL-8 response and the effect of brief hypoxia on other macrophage-derived chemokines and cytokines. We found that mRNA expression of several chemokines, in contrast to IL-8, were clearly downregulated by hypoxia. For example MIP-1 α , a CC chemokine with monocyte and neutrophil chemoattractant properties was inhibited by 2 hours hypoxia exposure. Similarly, mRNA expression of the archetypal early proinflammatory cytokine TNF- α was reduced under hypoxic conditions. These findings provide further evidence that hypoxia-induced transcription of these chemokines in macrophages is independent of NF- κ B. As with IL-8, the promoter regions of MCP-1, MIP- α , and TNF, all harbour functionally important NF- κ B binding sites. Hypoxia downregulates steady-state mRNA expression of these mediators without reducing NF- κ B DNA binding activity to a consensus probe. In contrast LPS has the capacity to upregulate a broad spectrum of inflammatory mediators, in association with increased NF- κ B activation.

These studies were prompted by our observation that raised intrapulmonary IL-8 levels were associated with ARDS progression in at-risk patients. In human macrophages acute hypoxia selectively upregulated IL-8 expression, in association with a novel pattern of transcription factor activation. However, the *in vitro* effects of hypoxia alone do not appear to be of sufficient magnitude to account for the differences in IL-8 observed in the clinical studies. Hypoxia is unlikely to represent the only relevant stimulus for IL-8 generation in the clinical setting. Rather several potentially proinflammatory stimuli including initial hypoxia, followed by reoxygenation/hyperoxia and pulmonary barotrauma during resuscitation and ventilation may create 'multiple-hits' to induce significant IL-8 generation and progression to ARDS. We are currently exploring this hypothesis in on-going studies.

Acknowledgments

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