Author's personal copy

EXPERIMENTAL CELL RESEARCH 318 (2012) 207–216

Available online at www.sciencedirect.com

SciVerse ScienceDirect

www.elsevier.com/locate/yexcr

Research Article

Changes in inflammatory gene expression induced by hyperbaric oxygen treatment in human endothelial cells under chronic wound conditions

Alexandra C. Kendall[a,b], *Jacqueline L. Whatmore*[a], *Lorna.W. Harries*[a], *Paul G. Winyard*[a], *Gary R. Smerdon*[b], *Paul Eggleton*[a,*]

[a]*Peninsula Medical School, University of Exeter, Exeter, Devon, EX1 2LU, UK*
[b]*Diving Diseases Research Centre, Tamar Science Park, Research Way, Plymouth, PL6 8BU, UK*

ARTICLE INFORMATION

Article Chronology:
Received 3 June 2011
Revised version received
7 October 2011
Accepted 23 October 2011
Available online 28 October 2011

Keywords:
Oxidative stress
Nitric oxide
Interleukin-8
Gene regulation
Chronic inflammation

ABSTRACT

Hyperbaric oxygen (HBO) therapy involves the inhalation of 100% oxygen, whilst inside a chamber at greater than atmospheric pressure. It is an effective treatment for chronic diabetic wounds, although the molecular mechanisms involved remain unclear. We hypothesised that HBO could alter inflammatory gene expression in human endothelial cells via a reactive oxygen/nitrogen species-mediated pathway. Endothelial cells were exposed to a chronic wound model comprising hypoxia (2% O_2 at 1 atmosphere absolute (ATA); PO_2 ~2 kPa) in the presence of lipopolysaccharide and TNF-α for 24 h, then treated with HBO for 90 min (97.5% O_2 at 2.4 ATA; PO_2 ~237 kPa). 5 h post-HBO, 19 genes involved in adhesion, angiogenesis, inflammation and oxidative stress were downregulated. Notably, only angiogenin gene expression, which promotes both angiogenesis and nitric oxide production (reflected by increased eNOS protein expression in this study), was upregulated. This led to a decrease in endothelial IL-8 mRNA and protein, which could help alleviate inflammatory processes during chronic wound healing. This was no longer evident 22.5 h post-HBO, demonstrating the importance of daily exposures in HBO treatment protocols. These studies indicate that elevated oxygen transiently regulates inflammatory gene expression in endothelial cells, which may enhance chronic wound healing.

© 2011 Elsevier Inc. All rights reserved.

Introduction

Chronic wounds are a huge burden on health services and the economy. In 2000, it was estimated that between three and six million people in the United States suffered from one of the three main types of chronic wound – a diabetic, venous or pressure ulcer, and that the total cost of wound care was around $3 billion per year [1]. As the population continues to age, this problem will grow, as people over 65 years old account for around 85% of chronic wound patients [1,2]. In normal wound healing, the wound progresses through three main overlapping phases – an inflammatory phase, a proliferative phase and a remodelling phase. These phases have characteristic cell involvement and involve intercellular signalling between platelets, macrophages, lymphocytes, polymorphonuclear leukocytes (PMNs), keratinocytes and endothelial cells, and the release of growth factors and cytokines from these cells orchestrates the healing process itself [3]. Wounds that fail to progress successfully through the normal

* *Corresponding author at:* Peninsula College of Medicine and Dentistry, University of Exeter, Exeter, EX1 2LU, UK. Fax: +44 1392 722926.
E-mail address: paul.eggleton@pms.ac.uk (P. Eggleton).
Abbreviations: CW, chronic wound; PC, pressure control.

0014-4827/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexcr.2011.10.014

Author's personal copy

healing process become chronic wounds, and healing is typically interrupted in the inflammatory phase [1].

Hypoxia is a key feature of chronic wounds, where destruction of the vasculature in the wound area, and high oxygen consumption by cells such as leukocytes and fibroblasts, result in low oxygen levels [4]. The PO_2 in chronic wounds has been measured using invasive oxygen electrodes, and has been found to lie in the range ~0.67–2.67 kPa (compared with typical values of ~4.0–6.67 kPa in healthy tissue) [5]. This prolonged hypoxia impairs several processes involved in wound healing, including collagen synthesis and deposition, epithelialisation, and phagocytic activity [6–8]. Additionally, chronic wounds are typically colonised by numerous species of bacteria, particularly opportunistic pathogens, as the hypoxic environment allows the growth of anaerobic bacteria, comprising 49% of bacterial species in chronic wounds compared with just 36% in acute wounds [9]. The prolonged hypoxia and bacterial contamination in the chronic wound leads to the release of large amounts of inflammatory cytokines by endothelial and other cells, exacerbating and propagating inflammation [10].

In order to provide nutrition and oxygen to wound sites to aid repair, the formation of new blood vessels via the process of angiogenesis is critical. The proliferation and migration of endothelial cells during the development of new capillary vessels help restore an oxygen supply to the ischaemic tissue. Understanding the molecular mechanisms that regulate wound healing in an inflammatory environment is essential if we are to provide new and more efficient treatment for chronic wounds.

HBO is an approved adjunctive therapy for the treatment of chronic diabetic wounds, used in conjunction with conventional treatments, including antibiotics, topical dressings, correction of vascular problems and debridement of tissue, when these latter treatments alone have proved ineffective. HBO involves the intermittent inhalation of 100% oxygen whilst inside a treatment chamber at a pressure greater than one ATA [11]. Breathing HBO forces high levels of oxygen to dissolve in the patient's plasma, which increases oxygen delivery to hypoxic wound tissue, where large intercapillary distances limit the diffusion of haemoglobin-bound oxygen. Oxygen dissolved in the plasma can diffuse further into the hypoxic tissue, raising wound oxygen levels from 0.67–2.67 kPa to 133.3–226.6 kPa [5,12]. A number of studies have provided evidence supporting the use of HBO in the treatment of chronic diabetic wounds, and an example of a wound treated successfully with HBO is shown in Fig. 1. HBO treatment has been shown to cause significant decreases in wound size area compared to control subjects [13] and more rapid healing rates [14], and a recent double-blinded placebo-controlled trial provided strong evidence to support the use of HBO in selected patients with chronic diabetic foot ulcers [15]. Despite these empirical observations, very few studies have investigated the effect of HBO on inflammatory gene expression over time in human endothelial or other cells at the site of wound damage. To address this lack of knowledge, we established a wound model employing human endothelial cells cultured under hypoxic conditions and exposed to tumour necrosis factor-alpha (TNF-α) and bacterial lipopolysaccharide (LPS), similar to the conditions observed in chronic wound sites. We monitored the real time expression of 92 pro- and anti-inflammatory genes under the wound model conditions pre- and post-HBO treatment. In addition, we focussed our attention on a number of gene products that appeared to be affected by HBO treatment in order to understand the wound healing processes that occur following HBO treatment.

Materials and methods

Reagents

Ham's F-12 medium and fetal bovine serum (FBS) were purchased from Lonza. Endothelial cell growth supplement (ECGS), collagenase, gentamicin, LPS and horseradish peroxidase (HRP)-conjugated polyclonal goat anti-rabbit secondary antibody were purchased from Sigma-Aldrich UK. TNF-α was purchased from the NIBSC. Gas mixes were purchased from BOC. RNeasy® Mini Kit, RNA stabilisation reagent, TURBO DNA-*free*™ kit, TaqMan® Reverse Transcription Reagents kit, custom TLDA plates, RNase-free water and TaqMan® Universal PCR Master Mix were purchased from Applied Biosystems. Rabbit polyclonal anti-human eNOS antibody was purchased from Abcam. IL-8 sandwich ELISA kit was purchased from eBioscience, Inc.

Cell culture and viability

Human umbilical cords were obtained within 24 h of birth from normal pregnancies. The anonymous collection of umbilical cords for this project was approved by the North and East Devon

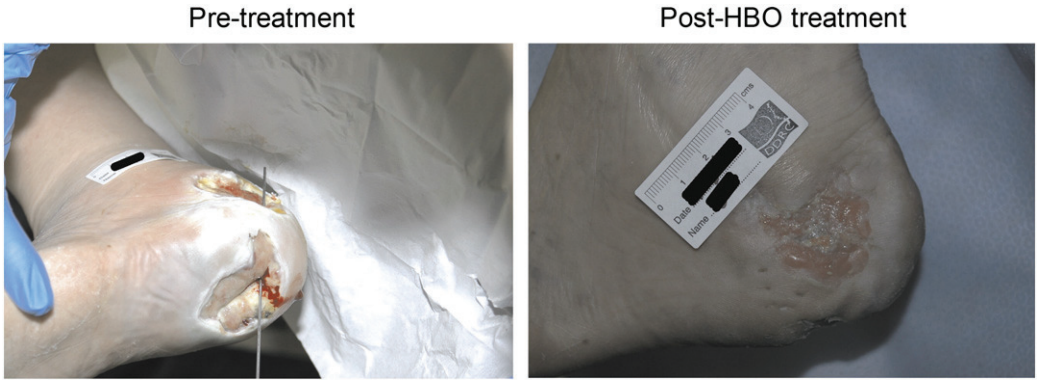

Fig. 1 – HBO is an effective treatment for chronic diabetic wounds. An example of a wound is shown before the initiation of HBO treatment, and five months post-treatment.

Author's personal copy

Medical Research Ethics Committee. Human umbilical vein endothelial cells (HUVEC) were isolated from the vein by collagenase (0.3 mg/ml) digestion [16] and cultured in Ham's F-12 medium containing 20% (v/v) FBS, 50 μg/ml gentamicin, and 20 μg/ml ECGS at 37 °C in 5% CO_2/air. The identity of endothelial cells in culture was confirmed by the presence of the typical "cobblestone" morphology and by the expression of von Willebrand factor and PECAM-1, as determined by immunocytochemical staining.

The effect of normoxia, hypoxia and HBO on endothelial cell viability was assessed by the well established techniques of trypan blue exclusion and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Endothelial cell viability was also assessed through microscopic observation of various morphological changes that typically occur during apoptosis or necrosis. In addition, flow cytometry was employed to monitor FITC-annexin V binding to phosphatidylserine (PS) on the surface of apoptotic cells that were exposed to normoxic and chronic wound conditions with and without HBO-treatment.

In vitro *chronic wound conditions and HBO/pressure control treatment*

Endothelial cells were exposed to *in vitro* chronic wound (CW) conditions by culturing them for 24 h with LPS from *Pseudomonas aeruginosa* (0.5 μg/ml) and TNF-α (1 ng/ml) in hypoxia (2% O_2 at 1 atmosphere absolute (ATA); PO_2 ~2 kPa) at 37 °C. Cells were exposed to HBO (97.5% O_2 at 2.4 ATA; PO_2 ~237 kPa) or pressure control (PC) conditions (0.83% O_2 at 2.4 ATA; PO_2 ~2 kPa) for 90 min in separate pressure vessels at 37 °C.

Adhesion of PMNs to an endothelial monolayer under flow

PMN adhesion to endothelial cells was monitored at physiological shear stress using a commercial flow system (Thistle Scientific, UK). PMN suspensions (1×10^6 cells/ml in RPMI containing 10% (v/v) FBS, 25 mM HEPES and 2 mM L-glutamine) were flowed over a monolayer of (passage 4 or less) HUVECs at a shear stress of 1 dyn/cm^2 for 4 min at 37 °C. The flow slides were examined using a Nikon Eclipse TS100 microscope, and photographs were taken of ten random fields/experimental condition. Each experiment was performed on three separate occasions.

RNA isolation and cDNA preparation for inflammatory gene QRT-PCR analysis

RNA expression was analysed as previously described [17,18]. Briefly, RNA was isolated from 2×10^6 HUVEC per treatment condition using an RNeasy® Mini Kit, and supplemented with 10 μl RNA stabilisation reagent. Contaminating DNA was removed using a TURBO DNA-*free*™ kit. RNA concentration and purity were assessed using a Nanodrop 8000 (Thermo Scientific, Leicestershire, UK) and quality was assessed using electrophoresis. cDNA was synthesised from 2.5 μg RNA per sample using a TaqMan® Reverse Transcription Reagents kit.

QRT-PCR of inflammatory genes in endothelial cells under hypoxic, HBO and pressure control conditions

For each treatment condition, 92 genes of interest (Table 1) were screened using custom-made TLDA plates. Briefly, reaction mixtures were prepared by mixing 2 μl of each cDNA sample (containing 100 ng cDNA) with 48 μl RNase-free water and 50 μl TaqMan® Universal PCR Master Mix, before 100 μl reaction mixture was added to each loading port on the array. Four samples were analysed per plate. Samples were amplified using the 7900HT Fast Real-Time PCR System (Applied Biosystems, Warrington, UK) with the following amplification conditions: 2 min at 50 °C (to activate uracil *N*-glycosylase), 10 min at 94.5 °C (for activation), and 50 cycles of 30 s at 97 °C (denaturation) and 1 min at 59.7 °C (annealing and extension). Gene expression data were analysed using relative quantification software, SDS 2.3 (Applied Biosystems, Warrington, UK) and the comparative threshold cycle method (ΔΔCt). Data were normalised against the average expression of four endogenous control genes (eukaryotic 18S RNA – *18S*, beta actin – *ACTB*, beta-2-microglobulin – *β2M* and glyceraldehye-3-phosphate dehydrogenase – *GAPDH*). Quality control was achieved by comparison of the quadruplicate crossing points for each gene per sample. At each time-point, gene expression levels of the HBO- or pressure-treated samples were normalised against those of chronic wound-treated cells.

Measurement of eNOS expression and production of NO_2^- (nitrite) and NO_3^- (nitrate)

HUVEC were treated as above and collected immediately, 5 h and 22.5 h post-hyperbaric treatment. Cells were analysed for eNOS expression by western blotting as follows: total protein samples were prepared by cell lysis in sample buffer containing 62.5 mM Tris HCl (pH 6.8), 2% (w/v) SDS, 25% (v/v) glycerol, 0.01% bromophenol blue and 50 mM DTT. Samples were heated at 100 °C for 5 min and 25 μg protein per sample was separated by SDS-PAGE and transferred to PVDF membrane at 60 V for 1 h. Blots were blocked for 1 h with 5% (w/v) milk powder (Marvel) in PBS containing 0.02% Tween-20 (PBS-T). Blots were then incubated with rabbit anti-human eNOS primary antibody diluted 1:1000 in PBS-T, overnight at 4 °C with gentle agitation. Blots were washed three times for 5 min in PBS-T, and then incubated with an HRP-conjugated goat anti-rabbit secondary antibody diluted 1:5000 in blocking buffer for 1 h at room temperature. Blots were washed six times for 5 min in PBS-T before bands were detected and their densities quantified using enhanced chemiluminescence detection on a Chemidoc XRS system with Quantity One software (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). Blots were stripped and reprobed for actin to assess protein loading.

To screen for stable end products of NOS activity, NO_2^- and NO_3^- in HUVEC cell culture medium were analysed using a chemiluminescence-based $^{\cdot}NO$ analyser (Sievers 280i $^{\cdot}NO$ analyser, GE Analytical Instruments, Boulder, Colorado). Samples were deproteinised according to the method of Higuchi and Motomizu [19]. Briefly, 100 μl of sample was diluted with 200 μl H_2O, then mixed with 300 μl 0.3 M NaOH and incubated for 5 min at room temperature. 300 μl 5% (w/v) $ZnSO_4$ was added and the mixture was vortexed then incubated for 10 min at room temperature. Finally, the mixture was centrifuged at 10,000×*g* for 15 min and the supernatant was collected for analysis as previously described [18]. NO_2^- was reduced to $^{\cdot}NO$ in 0.3 M NaI in acetic acid at room temperature, and NO_3^- was reduced to $^{\cdot}NO$ in VCl_3 in HCl, at 95 °C. Chemiluminescence was produced by a reaction between $^{\cdot}NO$ and ozone (O_3), creating electronically-excited nitrogen dioxide (NO_2) that was then detected by a photomultiplier tube. Values for NO_2^- and NO_3^-

Author's personal copy

Table 1 – Target genes screened in endothelial cells after exposure to chronic wound conditions pre- and post-HBO treatment.

Category	Protein (gene) name	General functions
Adhesion molecules	ICAM-1 (*ICAM1*); VCAM-1 (*VCAM1*); E-selectin (*SELE*); P-selectin (*SELP*); PECAM-1 (*PECAM1*); Mucosal addressin cell adhesion molecule-1 (*MADCAM1*); Junctional adhesion molecule 1 (*F11R*); JAM2 (*JAM2*); JAM3 (*JAM3*); Fibronectin 1 (*FN1*); Lymphocyte-specific protein 1 (*LSP1*); CD99 (*CD99*); Endoglin (*ENG*)	Adhesion molecules involved in leukocyte recruitment/ attachment
Angiogenic growth factors	VEGF A (*VEGFA*); VEGF B (*VEGFB*); VEGF C (*VEGFC*); Angiogenin (*ANG*); Angiopoietin 1 (*ANGPT1*); Angiopoietin 2 (*ANGPT2*); Angiopoietin 4 (*ANGPT4*); Connective tissue growth factor (*CTGF*); EGF-like repeat and discoidin I-like domain-containing protein 3 (*EDIL3*); Thymidine phosphorylase – endothelial cell growth factor 1 (*TYMP*)	Important growth factors promoting cell proliferation, blood vessel growth and permeabilisation
Angiogenesis targets	Fms-related tyrosine kinase 1 – VEGF receptor 1 (*FLT1*); Kinase insert domain receptor – VEGF receptor 2 (*KDR*); TEK tyrosine kinase, endothelial (*TEK*); Tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (*TIE1*)	Receptors for growth factors leading to vascular development and permeability
Angiogenesis inhibitors	A disintegrin and metalloproteinase with thrombospondin motifs 1 (*ADAMTS1*); TNF (ligand) superfamily – member 15 (*TNFSF15*); Vasohibin 1 (*VASH1*); Tissue inhibitor of metalloproteinases 2 (*TIMP2*)	Can act as angiogenic inhibitors
Tissue remodelling	MMP-2 (*MMP2*); MMP-9 (*MMP9*); TIMP1 (*TIMP1*); TIMP3 (*TIMP3*); TIMP4 (*TIMP4*)	Collagen degraders and promoters of cell proliferation and cell remodelling
Apoptosis	Caspase-1 (*CASP1*); Caspase-2 (*CASP2*); Caspase-3 (*CASP3*); Caspase-6 (*CASP6*); Caspase-7 (*CASP7*); Caspase-8 (*CASP8*); Caspase-9 (*CASP9*); Caspase-10 (*CASP10*); p53 (*TP53*)	Regulation of apoptosis
Oxygen response and redox signalling	HIF-1α (*HIF1A*); HIF-1β (*ARNT*); Endothelial PAS domain protein 1 – HIF-2α (*EPAS1*); HIF-3α (*HIF3A*); Haem-oxygenase-1 (*HMOX1*); Peroxiredoxin 1 (*PRDX1*); Peroxiredoxin 2 (*PRDX2*); Peroxiredoxin 6 (*PRDX6*); Glutathione peroxidase 1 (*GPX1*); CuZnSOD (*SOD1*); MnSOD (*SOD2*); Catalase (*CAT*); Xanthine dehydrogenase (*XDH*); Glutathione S-transferase alpha 1 (*GSTA1*); Sulfiredoxin 1 homolog (*SRXN1*); Thioredoxin (*TXN*); NADPH oxidase 4 (*NOX4*); Glyoxalase 1 (*GLO1*)	Regulators of oxidative stress
Nitric oxide synthesis	eNOS (*NOS3*); iNOS (*NOS2*); nNOS (*NOS1*); Nitric oxide synthase trafficker (*NOSTRIN*); Nitric oxide synthase interacting protein (*NOSIP*)	Involved in the production and regulation of the bioactive molecule nitric oxide
Cytokines and receptors	TNF-α (*TNF*); TNF receptor 1 (*TNFRSF1A*); TNF receptor 2 (*TNFRSF1B*); IL-8 (*IL8*)	Proinflammatory mediators and cell recruitment
Prostaglandin metabolism	Prostaglandin endoperoxide synthase 1 (*PTGS1*); Prostaglandin endoperoxide synthase 2 (*PTGS2*)	Regulators of angiogenesis and prostaglandin biosynthesis
Inflammatory mediators	Phospholipase A2, group IIA (*PLA2G2A*); Phospholipase A2, group IID (*PLA2G2D*); Annexin A1 (*ANXA1*); Bradykinin receptor B1 (*BDKRB1*); Calreticulin (*CALR*); Calreticulin 3 (*CALR3*)	Regulators of pro- and anti-inflammatory processes
NF-κB	NF-κB, subunit 1 (*NFKB1*); NF-κB, subunit 2 (*NFKB2*); c-Jun (*JUN*); c-Fos (*FOS*)	Pro and anti-inflammatory gene expression regulators
MAP kinases	MAPK1 (*MAPK1*); MAPK3 (*MAPK3*); MAPK8 (*MAPK8*); MAPK9 (*MAPK9*); MAPK11 (*MAPK11*); MAPK12 (*MAPK12*); MAPK13 (*MAPK13*); MAPK14 (*MAPK14*)	Regulators of phosphorylation-dependent cellular processes
Endogenous controls	Eukaryotic 18S RNA (*18S*); Beta actin (*ACTB*); Beta-2-microglobulin (*β2M*); Glyceraldehye-3-phosphate dehydrogenase (*GAPDH*)	Essential 'housekeeping' products

concentration were measured in duplicate and calculated from standard curves generated using known concentrations of $NaNO_2$ and $NaNO_3$, respectively.

IL-8 measurement

The concentration of IL-8 in culture medium collected from HUVEC under the different treatment conditions was quantified using a commercial IL-8 sandwich ELISA. Briefly, HUVEC were treated in gelatin-coated 96-well plates and cell culture medium was collected. High affinity 96-well plates (Corning Costar 9018) were coated with 100 μl per well of capture antibody in PBS overnight at 4 °C. Wells were washed five times with 250 μl per well PBS-T then blocked with 200 μl per well blocking buffer. Wells were washed five times and 100 μl standard or sample was added per well and incubated for 2 h at room temperature. Wells were washed five times then 100 μl biotinylated detection antibody was added per well and incubated for 1 h at room temperature. Wells were washed five times then 100 μl avidin-HRP was added and incubated for 30 min at room temperature. Wells were washed seven times then 100 μl TMB substrate solution was added per well and incubated for 15 min at room temperature. Reactions were stopped with 50 μl per well of 2 M H_2SO_4 and plates were read at 450 nm using a FLUOstar OPTIMA platereader (BMG Labtech Ltd, Aylesbury, UK).

Data analysis

Differences in quadruplicate measurements of gene expression data between different treatment conditions were analysed using the Mann Whitney U test. Levels of eNOS were expressed as a percentage of the data for the unstimulated cells at 0 h post-treatment before analysis with the Mann Whitney U test. Total

Author's personal copy

nitrite and nitrate levels in different treatment groups were also compared using the Mann Whitney U test. In all cases, P values <0.05 were considered statistically significant.

Results

The effect of chronic wound conditions and HBO on endothelial cell viability

Endothelial cell viability was not affected by changes in oxygen conditions (hypoxia or HBO), as measured by trypan blue, MTT, and assessment of morphological changes (Figs. 2A and C). Flow cytometric analysis of FITC-annexin V binding was employed to examine the effects of treatment with the chronic wound conditions, with or without HBO, on endothelial apoptosis revealed no differences in cell viability in cells that were either left unstimulated, or were treated with the chronic wound conditions then HBO (Fig. 2B).

Changes in endothelial cell inflammatory gene expression following treatment with HBO

The genes that demonstrated significant changes in mRNA expression 5 h after HBO treatment are presented in Fig. 3A. At 5 h post-hyperbaric treatment, samples collected from the CW+PC-treated cells did not provide sufficient expression data for enough genes to allow comparison with other treatment conditions. For this reason, these data were excluded from further analysis. At 22.5 h post-treatment, data for both HBO- and PC-treated cells were obtained, and those genes that demonstrated significant differences in mRNA expression from CW-treated cells are presented in Fig. 3B. Of the 20 genes that demonstrated significant changes in mRNA expression 5 h after HBO treatment, 19 demonstrated reduced expression (Fig. 3A). Three pro-inflammatory adhesion molecules, P-selectin, PECAM-1 and CD99, all of which are involved in neutrophil adhesion and transmigration, had reduced mRNA expression. The mRNA expression

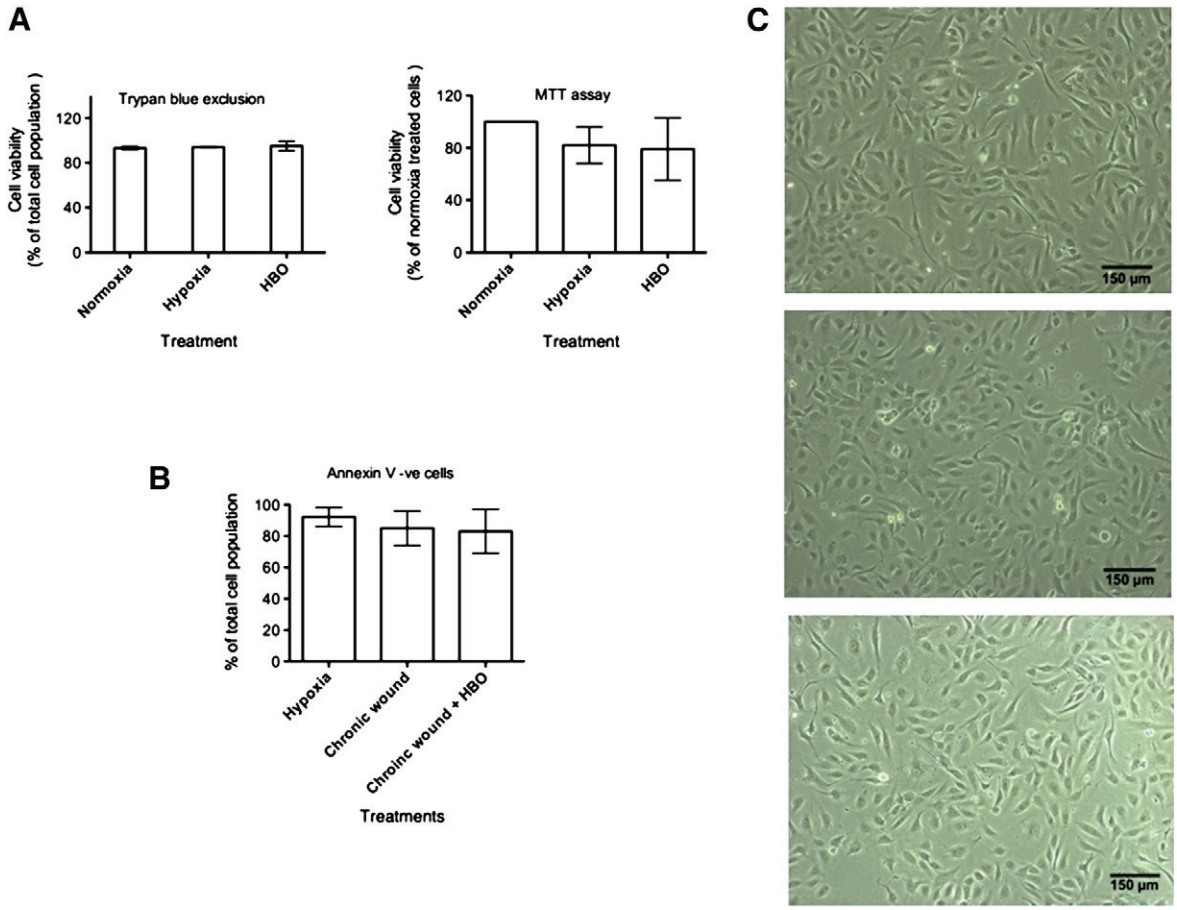

Fig. 2 – Effect of variable oxygen and chronic wound conditions on cell viability, apoptosis and morphology. HUVEC from three different cell populations were treated with either normoxia (21% O_2, 5% CO_2 at 1 ATA) for 5 h, hypoxia (2% O_2, 5% CO_2 at 1 ATA) for 5 h or HBO (95% O_2, 5% CO_2 at 2.4 ATA) for 90 min followed by 3.5 h in normoxia. (A) Trypan blue dye exclusion assay (left panel) and MTT assay (right panel) were then used to measure cell viability. (B). FITC-Annexin V binding was assessed in HUVEC from three different cell populations that were either left untreated or exposed to hypoxia in the presence of 0.5 µg/ml LPS and 1 ng/ml TNF-α for 24 h (chronic wound conditions). Cells treated with the chronic wound conditions were then either left in hypoxia at 1 ATA for a further 24 h, given a 90 min HBO treatment at 2.4 ATA then returned to hypoxia for 22.5 h. (C) Light microscopy assessment of cell morphology revealed no significant changes between normoxia-treated cells (upper panel) or after hypoxia (middle panel) or HBO treatment (lower panel).

Author's personal copy

of two other genes that have been implicated in neutrophil recruitment was also significantly reduced by HBO treatment, the pro-inflammatory cytokine IL-8, and the anti-inflammatory phospholipid-binding protein, annexin A1.

Several genes with roles in angiogenesis and tissue remodelling also demonstrated significant changes in mRNA expression. Notably, the pro-angiogenic growth factor angiogenin was the only gene that demonstrated a significant increase in mRNA expression 5 h post-HBO, which remained elevated 17.5 h later (Fig. 3B). Another pro-angiogenic growth factor, CTGF, demonstrated decreased expression 5 h-post HBO. Interestingly, both TIE-2 (tyrosine kinase with immunoglobulin-like and EGF-like domains-2; *TEK*), the pro-angiogenic angiopoietin 1 receptor, and its inhibitor, angiopoietin 2, demonstrated decreased mRNA

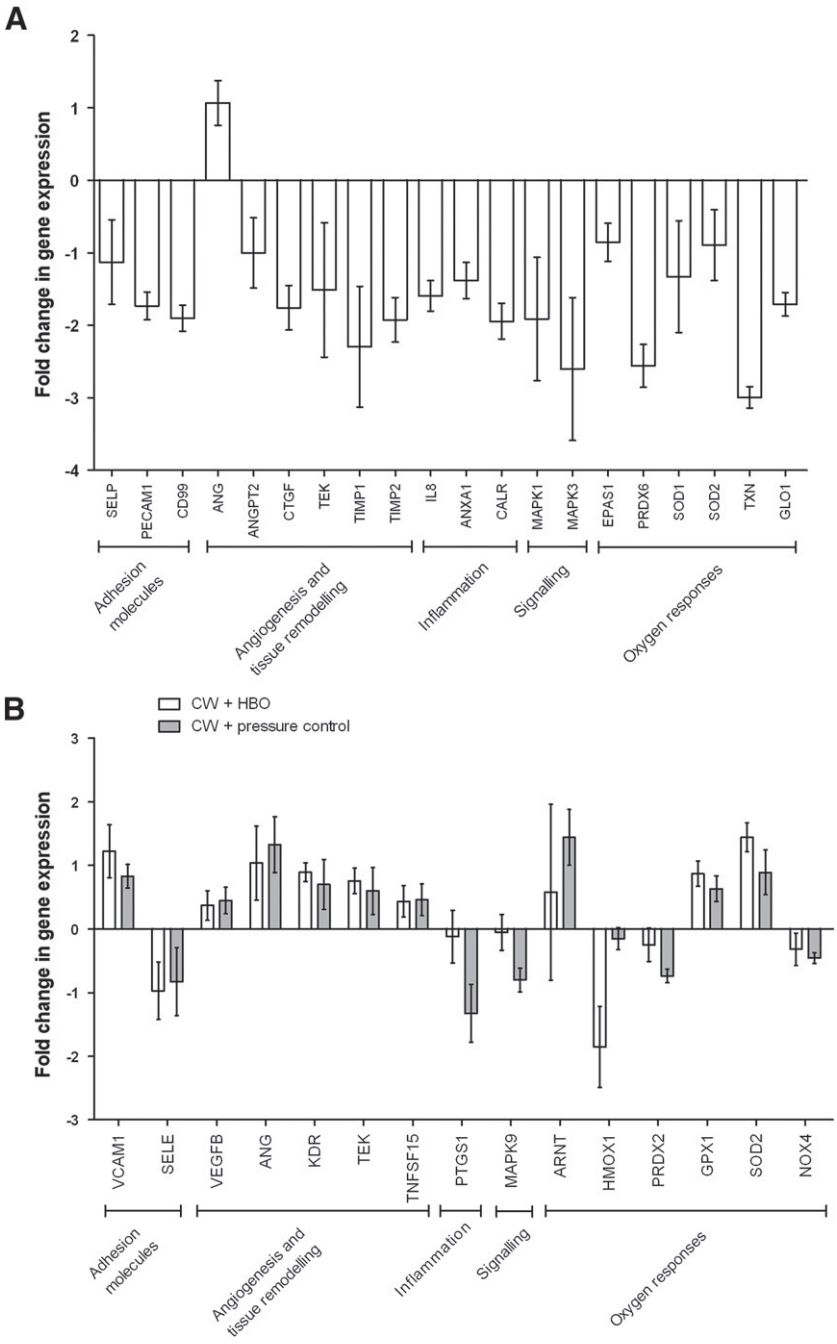

Fig. 3 – Changes in inflammatory gene expression in HBO-treated endothelial cells. HUVEC were treated with chronic wound (CW) conditions for 24 h, then given a 90 min treatment with HBO or pressure control (PC) conditions. mRNA was harvested 5 h and 22.5 h post-treatment and analysed for changes in gene expression. (A) In CW-treated HUVEC, 20 genes demonstrated statistically significant changes in mRNA expression 5 h post-treatment with HBO. (B) In CW-treated HUVEC, 15 genes demonstrated significant changes in mRNA expression 22.5 h post-treatment with HBO or the PC. Data for mRNA expression in HBO-treated cells are expressed as a fold-change in expression from CW-treated cells (mean ± S.D.).

Author's personal copy

expression. Two TIMPs, TIMP1, which can promote cell proliferation, and TIMP2, which inhibits endothelial cell proliferation, both demonstrated reduced expression 5 h post-HBO. Calreticulin (*CALR*), which has also been shown to increase proliferation of endothelial cells, demonstrated reduced mRNA expression 5 h after treatment with HBO.

Several genes involved in cellular oxygen responses and redox signalling also demonstrated significant changes, with HIF-2α (*EPAS1*), peroxiredoxin 6 (*PRDX6*), superoxide dismutase 1 (*SOD1*), superoxide dismutase 2 (*SOD2*), thioredoxin (*TXN*) and glyoxalase 1 (*GLO1*) all demonstrating significant reductions in mRNA expression following HBO treatment. By the second time-point, 22.5 h after hyperbaric treatment, the expression of all but one of these genes (*SOD2*) was no longer significantly different from that in CW-treated cells. The expression of *SOD2* mRNA was actually higher in HBO-treated cells at this time-point.

Some genes demonstrated significant changes in mRNA expression 22.5 h post-HBO treatment or the PC (Fig. 3B). Two of these genes were the adhesion molecules, VCAM-1, which demonstrated increased expression following either treatment, and E-selectin, which demonstrated decreased expression following either treatment. Both VEGF B and the VEGF receptor KDR (*VEGFR2*) were upregulated 22.5 h after treatment with HBO. TNF (ligand) superfamily member 15 (*TNFSF15*), an inhibitor of angiogenesis, demonstrated significantly increased mRNA expression 22.5 h after HBO treatment, but not PC treatment. Heme oxygenase-1 (HO-1; *HMOX1*) mRNA expression was reduced by HBO treatment, but not treatment with the PC, whilst glutathione peroxidase 1 (*GPX1*) expression was increased following treatment with either HBO or the PC.

A number of genes were unaffected by HBO treatment and remained at constant levels at the time points tested in this study (Table 2). Examples include endoglin (*ENG*), showing a lack of endothelial activation under HBO, and caspases-1, -6 and -8, demonstrating a lack of an HBO-mediated effect on apoptosis.

IL-8 release

As IL-8 mRNA expression in CW-treated cells demonstrated a clear response to HBO, with a significant decrease in expression 5 h post-treatment that was resolved at 22.5 h post-treatment (Fig. 4A), we examined the release of IL-8 protein (Fig. 4B). The concentration of secreted IL-8 was measured in cell culture medium collected from HUVEC treated with CW conditions, with or without a 90 min HBO treatment, 5 h and 22.5 h post-HBO treatment. Cells treated with chronic wound conditions released 170.0 ± 16.0% (mean ± S.D.) of the amount released by unstimulated cells, but this increased release was significantly reduced by a single HBO treatment, as CW-treated cells that also received HBO treatment released only 124.6 ± 23.8% of the amount released by unstimulated

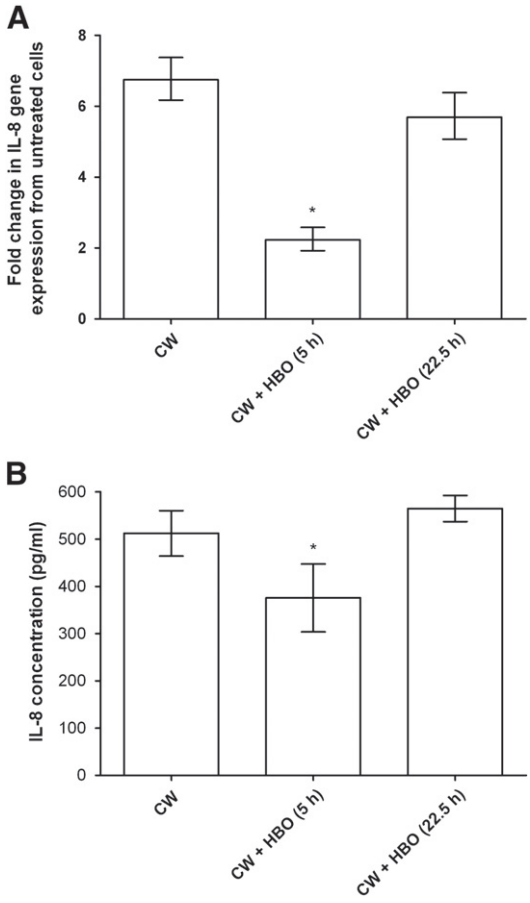

Fig. 4 – Effect of HBO on IL-8 mRNA and protein expression with time. HUVEC were treated with CW conditions for 24 h then given a 90 min treatment with HBO. mRNA and cell culture medium were collected 5 h and 22.5 h post-treatment and analysed for IL-8 gene expression and protein concentration, respectively. (A) IL-8 mRNA expression presented as mean fold changes ± S.D. in relative gene expression normalised to four control genes, and compared to unstimulated (US) cells. (B) IL-8 protein levels relative to US cells at the same time points. Data expressed as the mean ± S.D. (N = 3). *P < 0.05 *versus* CW (5 h).

Table 2 – Target genes that were unaffected by HBO (5 h and 22.5 h post-treatment) or pressure control treatment (22.5 h post-treatment).

Category	Protein (gene) name
Adhesion molecules	Junctional adhesion molecule 1 (*F11R*); Fibronectin 1 (*FN1*); Endoglin (*ENG*)
Angiogenic growth factors	VEGF A (*VEGFA*); EGF-like repeat and discoidin I-like domain-containing protein 3 (*EDIL3*)
Angiogenesis targets	Tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (*TIE1*)
Apoptosis	Caspase-1 (*CASP1*); Caspase-6 (*CASP6*); Caspase-8 (*CASP8*)
Oxygen response and redox signalling	HIF-1α (*HIF1A*); Peroxiredoxin 1 (*PRDX1*); Catalase (*CAT*)
NF-κB	NF-κB, subunit 1 (*NFKB1*); c-Jun (*JUN*)
MAP kinases	MAPK8 (*MAPK8*)

Author's personal copy

(US) cells. The effect of HBO on IL-8 secretion was no longer evident 22.5 h post-treatment, as IL-8 levels had returned to 163.2 ± 8.0% of the amount released by US cells. When freshly-isolated, untreated PMNs were flowed over untreated endothelial cells, very few neutrophils adhered (Fig. 5A). However, when the endothelial cells were treated for 48 h in chronic wound conditions, the number of PMNs adhering significantly increased ($P = 0.037$; Fig. 5B), compared to adherence to untreated endothelial cells. A 90 min HBO treatment of the endothelial cells, 24 h into treatment with the chronic wound conditions led to a significant reduction in the number of adherent PMNs compared to chronic wound conditions ($P < 0.05$; Fig. 5C).

eNOS protein expression and ˙NO release

Angiogenin mRNA expression was increased 5 h post-HBO treatment, and remained significantly high at 22.5 h post-treatment (Figs. 3A and B). Angiogenin is known to induce ˙NO synthesis in endothelial cells [20]. Since ˙NO is a key factor in angiogenesis we examined eNOS protein expression and the stable products of ˙NO production, nitrite and nitrate (Fig. 6). Total eNOS protein expression in HUVEC following treatment with CW conditions and HBO or the PC was analysed by western blotting, immediately, 5 h and 22.5 h post-hyperbaric treatment (Figs. 6A and B). Immediately post-treatment, cells that had been treated with the CW conditions (with or without a hyperbaric treatment) demonstrated an increase in the expression of eNOS compared to US cells. CW treatment alone caused a 3.2 ± 1.4% increase in eNOS expression. Cells that received an additional hyperbaric treatment (either HBO or the PC) showed a significantly greater increase in eNOS expression, with increases of 10.5 ± 0.9% and 8.9 ± 3.2%, respectively, compared with the expression in US cells (Fig. 6B). A similar pattern of eNOS expression was observed at 5 h and 22.5 h post-treatment, although the differences between treatments were not statistically significant.

Nitrite and nitrate concentrations in medium collected from these cells were assessed using a chemiluminescence-based ˙NO analyser. Total NO_2^- plus NO_3^- levels are shown in Fig. 6C, and demonstrate no statistically significant differences between treatments.

Discussion

This study has shown that a single HBO treatment can significantly alter the expression of a number of genes in endothelial cells specifically involved in inflammation and wound healing. Interestingly, HBO treatment appears to alter the expression of different genes, 5 h and/or 22.5 h post-HBO treatment. 5 h post-HBO treatment a number of genes that are involved in adhesion, angiogenesis, cell recruitment, signalling and redox regulation appeared to be down-regulated, while only one gene (angiogenin) increased over this time period. The cellular physiology of angiogenin is poorly understood, but angiogenin is an angiogenic factor that is essential for cell proliferation, and plays a role in the synthesis of the biological transmitter ˙NO [20]. ˙NO is known to play a critical role in vascular physiology and pathology, but has a very short half-life and is difficult to measure in real time [21]. In this study, we measured the eNOS protein expression immunochemically and used a sensitive chemiluminescence technique to measure nitrite/nitrate endpoint products of ˙NO synthesis. A significant increase in eNOS expression was observed with CW treatment, and this was further increased immediately post-HBO and PC treatment. This trend continued at both 5 h and 22.5 h post-treatment, with a lack of statistical significance. The upregulation of angiogenin gene expression and eNOS protein expression supports the observations of previous work [20]. In our study it would appear that this process is influenced by HBO and/or increased pressure. However we were unable to demonstrate an increase in nitrite/nitrate concentrations. One explanation for this may be that nitrite and nitrate are formed by other pathways, including those catalysed by iNOS, nNOS and xanthine oxidoreductase, which may confound the changes in nitrite/nitrate that are attributable to changes in eNOS activity [22].

Another notable gene that was down-regulated 5 h after a single HBO treatment was the pro-inflammatory cytokine and chemoattractant – *IL8*. A number of studies have provided evidence linking ˙NO production to the regulation of IL-8. An early study suggested that treatment of endothelial cells with ˙NO inhibitors

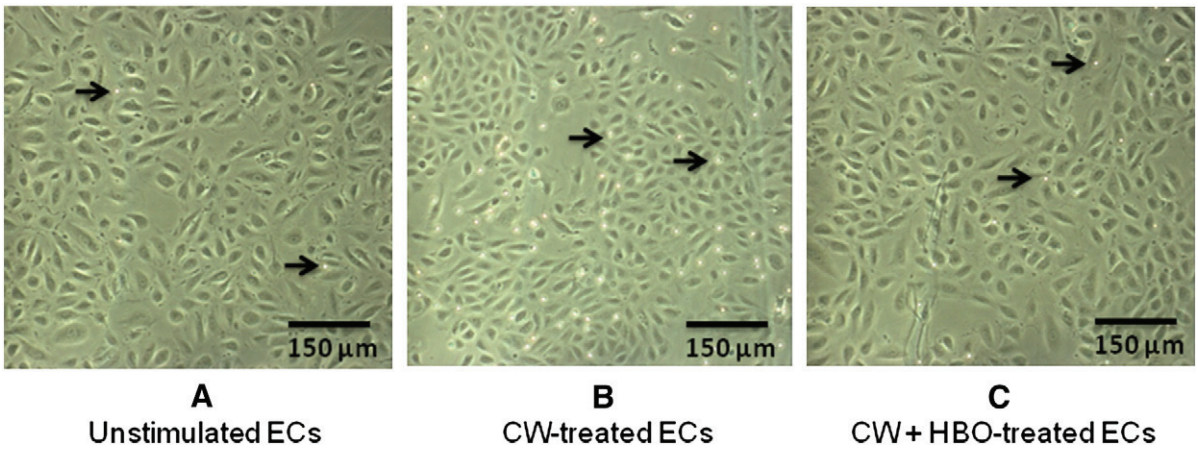

Fig. 5 – Effect of HBO on PMN adhesion to endothelial cells. Freshly isolated PMNs were flowed over A) unstimulated endothelial cells, B) endothelial cells treated in chronic wound conditions for 48 h or C) endothelial cells treated in chronic wound conditions, given a 90 min HBO treatment, then returned to chronic wound conditions for a further 22.5 h. PMNs, were flowed over endothelial cells for 4 min at 1 dyn/cm^2. Non-adherent PMNs were removed by washing, and adherent PMNs were counted under phase-contrast microscopy. Examples of adherent PMNs are highlighted with arrows.

Author's personal copy

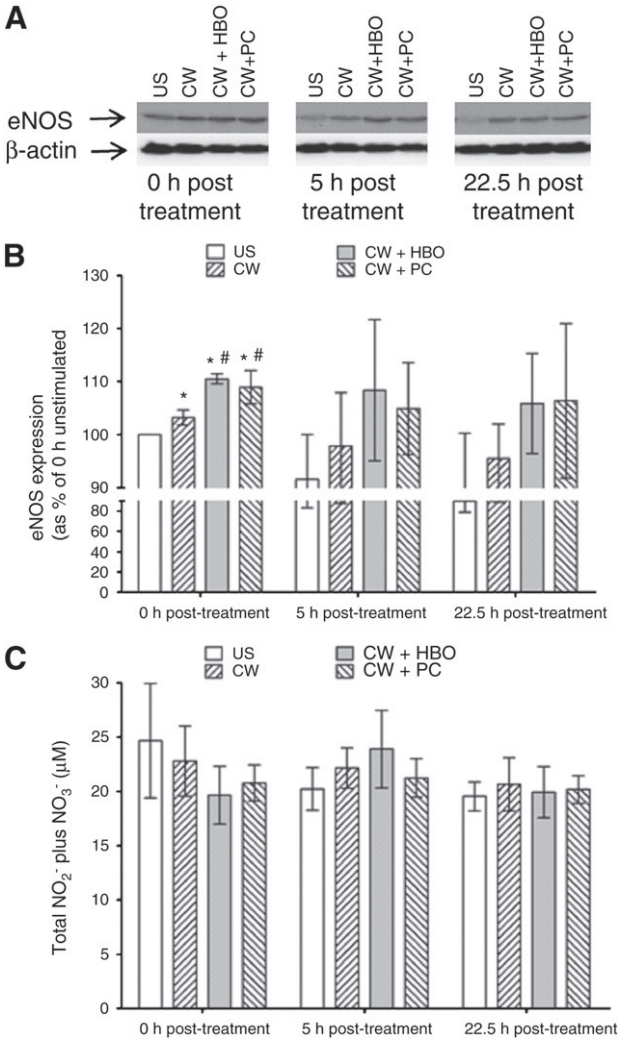

Fig. 6 – Effect of HBO on eNOS protein expression and total nitrate/nitrite production in CW-treated human endothelial cells. HUVEC were treated for 24 h with CW conditions, then given a 90 min HBO treatment. Cells and culture medium were harvested immediately, 5 h and 22.5 h post-treatment and analysed for eNOS total protein and nitrite/nitrate levels, respectively. (A) Western blots of eNOS protein levels of HUVEC whole cell lysates obtained from unstimulated (US) cells or after chronic wound (CW), CW + HBO or CW + PC treatment and immediately, 5 h and 22.5 h post-treatment (upper panels), together with β-actin loading controls (lower panels). (B) Following densitometry and normalisation against β-actin, total eNOS expression was expressed as a percentage of that in US cells at 0 h post-treatment (mean ± S.D., N = 3). *P = 0.037 *versus* unstimulated, #P = 0.0495 *versus* chronic wound conditions. (C) NO_2^-/NO_3^- levels in cell culture medium collected from the cells were measured in duplicate using a chemiluminescence-based ˙NO analyser. Data expressed as the mean ± S.D. (N = 3).

or ˙NO donors decreased or increased IL-8 levels, respectively [23]. However, our own data suggest that increased eNOS expression correlates with reduced IL-8 expression at the mRNA level and protein level in endothelial cells treated with HBO. This is in agreement with later studies, which demonstrated that increased ˙NO significantly reduces IL-8 mRNA in endothelial cells, by inhibiting nuclear factor-kappaB (NF-κB) binding to the transcriptional start site in the IL-8 promoter [24], and that inhaled ˙NO can reduce the IL-8 protein concentration in blood and bronchial lavage [25]. The multiple pathways by which IL-8 production can be regulated may explain the conflicting results in the literature. For example, ˙NO released from the ˙NO donor, *S*-nitroso-*N*-acetyl-penicillamine (SNAP), can increase HO-1 activity, which in turn up-regulates VEGF production, which in turn induces IL-8 production [26]. In our study, HBO was not shown to affect VEGF mRNA production by endothelial cells at 5 h post-treatment. However, an isoform of VEGF (*VEGFB*) was significantly upregulated 22.5 h post-HBO treatment, which paralleled an increase in IL-8 levels back to levels seen prior to HBO treatment. The ability of HBO treatment to reduce IL-8 production at the mRNA expression and protein secretion level may explain some of the anti-inflammatory effects of HBO during wound healing. In this study, IL-8 production by endothelial cells at the mRNA and protein level was reduced. We therefore looked at the ability of HBO treatment to alter PMN adhesion to endothelial cells under flow conditions. A single treatment of HBO to the endothelial cells reduced visibly the numbers of PMNs adhering to the endothelial cells. The reduction in neutrophil adhesion may partly be explained by the changes in IL-8 production observed in this study.

Our results suggest that HBO-mediated changes in gene expression are dynamic and transient. At 5 h post-HBO treatment, the majority of genes which were altered by HBO were downregulated (19 genes downregulated, one upregulated). However, 22.5 h post-HBO treatment, a greater number of genes appeared to be upregulated compared with CW conditions. It was notable that genes particularly beneficial in angiogenesis and tissue remodelling, together with antioxidant enzymes that help suppress ROS-mediated cellular damage, were significantly upregulated at the second time-point.

In summary, we have examined the effect of HBO treatment on endothelial cell gene expression under CW conditions for the first time. This has revealed that HBO activates a number of genes. Initially, HBO appears to promote angiogenin expression and eNOS activity, which correlates with a decrease in IL-8 at the mRNA and protein level. This may be important in the chronic wound scenario as IL-8 has a direct effect on endothelial cell survival and proliferation, as well as a key role as a pro-inflammatory chemoattractant. However 22.5 h post-HBO treatment, IL-8 levels begin to recover and a number of regulatory genes for endothelial cell survival and angiogenesis are activated. These responses may help to understand, in part, some of the molecular effects of HBO treatment in endothelial cells at wound sites, and may be useful to improve the clinical application of HBO for various inflammatory conditions.

Acknowledgments

This work was supported by a grant from the Diving Diseases Research Centre, Plymouth, UK [RR100098]. ACK was supported by a Peninsula College of Medicine and Dentistry Studentship awarded to PE, JLW, GRS and PGW. The funding bodies had no involvement in the conduct of the research or the preparation of the article.

Author's personal copy

REFERENCES

[1] N.B. Menke, K.R. Ward, T.M. Witten, D.G. Bonchev, R.F. Diegelmann, Impaired wound healing, Clin. Dermatol. 25 (2007) 19–25.
[2] R.F. Diegelmann, Excessive neutrophils characterize chronic pressure ulcers, Wound Repair Regen. 11 (2003) 490–495.
[3] V. Falanga, Wound healing and its impairment in the diabetic foot, Lancet 366 (2005) 1736–1743.
[4] A.A. Tandara, T.A. Mustoe, Oxygen in wound healing – more than a nutrient, World J. Surg. 28 (2004) 294–300.
[5] P.J. Sheffield, Measuring tissue oxygen tension: a review, Undersea Hyperb. Med. 25 (1998) 179–188.
[6] G.M. Gordillo, C.K. Sen, Revisiting the essential role of oxygen in wound healing, Am. J. Surg. 186 (2003) 259–263.
[7] M.A. Ngo, N.N. Sinitsyna, Q. Qin, R.H. Rice, Oxygen-dependent differentiation of human keratinocytes, J. Invest. Dermatol. 127 (2007) 354–361.
[8] H.W. Hopf, M.D. Rollins, Wounds: an overview of the role of oxygen, Antioxid. Redox. Signal. 9 (2007) 1183–1192.
[9] P.G. Bowler, B.J. Davies, The microbiology of infected and noninfected leg ulcers, Int. J. Dermatol. 38 (1999) 573–578.
[10] J.S. Lewis, J.A. Lee, J.C. Underwood, A.L. Harris, C.E. Lewis, Macrophage responses to hypoxia: relevance to disease mechanisms, J. Leukoc. Biol. 66 (1999) 889–900.
[11] C.L. Broussard, Hyperbaric oxygenation and wound healing, J. Vasc. Nurs. 22 (2004) 42–48.
[12] R.L. Williams, Hyperbaric oxygen therapy and the diabetic foot, J. Am. Podiatr. Med. Assoc. 87 (1997) 279–292.
[13] W.A. Zamboni, H.P. Wong, L.L. Stephenson, M.A. Pfeifer, Evaluation of hyperbaric oxygen for diabetic wounds: a prospective study, Undersea Hyperb. Med. 24 (1997) 175–179.
[14] L. Kessler, P. Bilbault, F. Ortega, C. Grasso, R. Passemard, D. Stephan, M. Pinget, F. Schneider, Hyperbaric oxygenation accelerates the healing rate of nonischemic chronic diabetic foot ulcers: a prospective randomized study, Diabetes Care 26 (2003) 2378–2382.
[15] M. Londahl, P. Katzman, A. Nilsson, C. Hammarlund, Hyperbaric oxygen therapy facilitates healing of chronic foot ulcers in patients with diabetes, Diabetes Care 33 (2010) 998–1003.
[16] E.A. Jaffe, R.L. Nachman, C.G. Becker, C.R. Minick, Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria, J. Clin. Invest. 52 (1973) 2745–2756.
[17] P. Eggleton, L. Harries, G. Alberigo, P. Wordsworth, N. Viner, R. Haigh, S. Donnelly, H. Jones, I. Chikanza, T. O'Conner, A. Thomson, P. Winyard, Changes in apoptotic gene expression in lymphocytes from rheumatoid arthritis and systemic lupus erythematosus patients compared with healthy lymphocytes, J. Clin. Immunol. 30 (2010) 649–658.
[18] J.M. Tarr, P.G. Winyard, B. Ryan, L.W. Harries, R. Haigh, N. Viner, P. Eggleton, Extracellular calreticulin is present in the joints of patients with rheumatoid arthritis and inhibits FasL (CD95L)-mediated apoptosis of T cells, Arthritis Rheum. 62 (2010) 2919–2929.
[19] K. Higuchi, S. Motomizu, Flow-injection spectrophotometric determination of nitrite and nitrate in biological samples, Anal. Sci. 15 (1999) 129–134.
[20] R. Trouillon, D.K. Kang, H. Park, S.I. Chang, D. O'Hare, Angiogenin induces nitric oxide synthesis in endothelial cells through PI-3 and Akt kinases, Biochemistry (Mosc) 49 (2010) 3282–3288.
[21] J.F. Arnal, A.T. Dinh-Xuan, M. Pueyo, B. Darblade, J. Rami, Endothelium-derived nitric oxide and vascular physiology and pathology, Cell. Mol. Life Sci. 55 (1999) 1078–1087.
[22] Z. Zhang, D. Naughton, P.G. Winyard, N. Benjamin, D.R. Blake, M.C. Symons, Generation of nitric oxide by a nitrite reductase activity of xanthine oxidase: a potential pathway for nitric oxide formation in the absence of nitric oxide synthase activity, Biochem. Biophys. Res. Commun. 249 (1998) 767–772.
[23] L.H. Villarete, D.G. Remick, Nitric oxide regulation of IL-8 expression in human endothelial cells, Biochem. Biophys. Res. Commun. 211 (1995) 671–676.
[24] A.A. Fowler III, B.J. Fisher, L.B. Sweeney, T.J. Wallace, R. Natarajan, S.S. Ghosh, S. Ghosh, Nitric oxide regulates interleukin-8 gene expression in activated endothelium by inhibiting NF-kappaB binding to DNA: effects on endothelial function, Biochem. Cell Biol. 77 (1999) 201–208.
[25] I. Moreno, A. Mir, R. Vicente, A. Pajares, F. Ramos, J.L. Vicente, M. Barbera, Analysis of interleukin-6 and interleukin-8 in lung transplantation: correlation with nitric oxide administration, Transplant. Proc. 40 (2008) 3082–3084.
[26] H.O. Pae, G.S. Oh, B.M. Choi, Y.M. Kim, H.T. Chung, A molecular cascade showing nitric oxide-heme oxygenase-1-vascular endothelial growth factor-interleukin-8 sequence in human endothelial cells, Endocrinology 146 (2005) 2229–2238.