Bach1 differentially regulates distinct Nrf2-dependent genes in human venous and coronary artery endothelial cells adapted to physiological oxygen levels

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The effects of physiological oxygen tension on Nuclear Factor-E2-Related Factor 2 (Nrf2)-regulated redox signaling remain poorly understood. We report the first study of Nrf2-regulated signaling in human primary endothelial cells (EC) adapted long-term to physiological O2 (5%). Adaptation of EC to 5% O2 had minimal effects on cell ultrastructure, viability, basal redox status or HIF1-α expression. Affymetrix array profiling and subsequent qPCR/protein validation revealed that induction of select Nrf2 target genes, HO-1 and NQO1, was significantly attenuated in cells adapted to 5% O2, despite nuclear accumulation and DNA binding of Nrf2. Diminished HO-1 induction under 5% O2 was stimulus independent and reversible upon re-adaptation to air or silencing of the Nrf2 repressor Bach1, notably elevated under 5% O2. Induction of GSH-related genes xCT and GCLM were oxygen and Bach1-insensitive during long-term culture under 5% O2, providing the first evidence that genes related to GSH synthesis mediate protection afforded by Nrf2–Keap1 defense pathway in cells adapted to physiological O2 levels encountered in vivo.

1. Introduction

The Nrf2 (NF-E2-related factor 2)/Keap1 (Kelch-like ECH-associated protein 1) pathway plays key role in cellular defenses against oxidative and environmental stress [1–4]. As a redox sensitive transcription factor, Nrf2 regulates endogenous antioxidant defenses, phase II metabolism, autophagy [5] and solute transporters [6]. Nrf2 is increasingly becoming recognized as a therapeutic target for the treatment of chronic diseases associated with oxidative stress [3,4,7–9]. The molecular mechanisms regulating Nrf2 signaling have predominantly been investigated in vitro using cell culture models exposed to atmospheric oxygen and identified multiple regulatory proteins which repress (Keap1, Bach1) or enhance (DJ-1, p62) Nrf2 activity [3,4,10]. As Nrf2 is activated under conditions of hypoxia (<1% O2) and hyperoxia [11–13], we investigated the regulatory mechanisms and downstream Nrf2 gene targets in human primary endothelial cells (EC) following long-term adaptation to physiological oxygen (5% O2). In vivo endothelial (EC) and other cell types reside in a
physiological O₂ environment, ranging from ~3 to 13%, while in culture cells are normally exposed to atmospheric O₂ levels (20.86% O₂ = 158 mmHg) [14]. Alterations in O₂ tension modulate cell function by affecting intracellular NAD(P)H levels, protein kinases, ion channels and antioxidant defenses [14–18]. Although studies in primary lymphocytes elegantly highlight the importance of studying cellular responses under physiological O₂ (5%) [19–21], the majority of studies have characterized signal transduction in cells cultured in ambient air, with only a few comparing responses after short-term adaptation to different O₂ tensions [13,20,22,23].

The present study provides the first detailed characterization of Nrf2-regulated redox signaling in human primary venous and arterial EC adapted long-term (5 days–2 weeks) to physiological oxygen (5% O₂). Critically, our study using an O₂-regulated work-station circumvented problems associated with re-oxygenation, enabling us to compare effects of short-term (24 h) versus long-term (5–14 days) adaptation to 5% O₂. A cell-penetrating phosphorescence, O₂-sensitive nanoparticle probe [24,25] enabled us to monitor real-time changes in cytosolic O₂ content. Using an Affymetrix array, we established that electroneutral stress induces Nrf2 signaling, with glutathione (GSH)-related genes insensitive to changes in O₂ tension. Although Nrf2 nuclear translocation was unaffected by physiological O₂ (5%), induction of select Nrf2 target genes (HO-1, NQO1) was diminished. We further report that basal expression of the Nrf2 repressor Bach1 is elevated in human umbilical vein (HUVEC) and coronary artery (HCAEC) EC adapted to 5% O₂. Knockdown of Bach1 restored HO-1 induction in EC cultured under 5% O₂ but had negligible effects on basal and electrophile induced GCLM expression, suggesting that under physiological O₂ levels encountered in vivo GSH-related genes transduce Nrf2-mediated cellular protection. Thus, investigating redox signaling in cells adapted long-term to physiological O₂ tension provides valuable insights for endothelial metabolism [26] and more importantly bench-to-bedside translation in cardiovascular and regenerative medicine [22,27].

2. Materials and methods

2.1. Reagents

Cell culture media, chemicals and reagents were obtained from Sigma-Aldrich (Dorset, UK) unless stated otherwise. Nucleosipin 96 RNA Kit was from Macherey-Nagel (Duren, Germany), High Capacity cDNA conversion kit from Applied Biosystems (Paisley, UK), SensiMix SYBR No-Rox PCR Kit from Bioline (London, UK), Trans-AM nuclear extraction and Nrf2 binding activity assay kit from Active Motif (Carlsbad, USA). Fluorescent superoxide indicator MitoSOX red (M36008) was from Invitrogen Molecular Probes (Carlsbad, USA), Antibodies against Nrf2 (SC-722, mol. wt. 110 kDa band), NQO1 (SC-16464), Lamin A/C (SC-6254), Bach-1 (SC-14700) from Santa Cruz (USA), Keap1 (DB612, #8047) and Dj-1 (#2134) from Cell Signaling (Massachusetts, USA), HO-1 (ab13243) and Nfkb/p65 (ab16502) from Abcam Cambridge, UK), HIF-1α (NB100–449) from Novus Biologicals (Abingdon, UK), β-tubulin (mab1864) and HRP-conjugated secondary goat anti-rabbit (AP132P), goat anti-rat (AP136P) and donkey anti-goat (AP234P HRP) secondary antibodies from Millipore (Billerica, USA). GCLM was a gift from Prof. Terrance J. Kavanagh (University of Washington, USA). Firefly luciferase/luciferin reaction mixture (FLE250) was from Sigma-Aldrich. Human coronary artery endothelial cells (HCAEC) were provided by PromoCell GmbH, Germany.

2.2. Endothelial cell culture under defined O₂ levels

Primary human umbilical vein endothelial cells (HUVEC) were cultured in M199 containing 10% fetal calf (FCS) and 10% newborn calf serum [28]. An endothelial phenotype was confirmed by a characteristic cobblestone morphology and positive immunostaining for von Willebrand factor and eNOS which was not altered during culture in 5% O₂ (data not shown). HUVEC and human coronary artery EC (HCAEC) were cultured in a standard incubator gassed with 5% CO₂ in air or an O₂-regulated workstation (Sci-tive, Baker-Ruskinn Company, Maine, USA) gassed with 5% O₂, 5% CO₂, 90% N₂ (75% humidity) with subculture and all treatments conducted within the workstation. All culture media were pre-equilibrated in either ambient air, 5% O₂ or 1% O₂ in the Sci-tive workstation prior to experimentation. Endothelial cells (passages 1–3) were pre-adapted to 5% O₂ for 1 or 5 days, unless stated otherwise. HUVEC were equilibrated in M199 containing 20% serum (15%EGS) for 24 h before treatment for a further 0–24 h with medium alone or containing diethylmaleate (DEM, 100 μmol L⁻¹), 4-hydroxynonenal (HNE, 20 μmol L⁻¹), sulforaphane (SFN, 2.5 μmol L⁻¹) or the NO donor (DETA NONOate, 500 μmol L⁻¹). HCAEC (Promocell C-12221, Germany) were pre-equilibrated in ECM (–growth factors, Promocell C-22020, Germany). For transfection, Bach1 or Nrf2 knockdown was achieved by transfecting HUVEC with 40 pmol/24 well scramble, Bach1 or Nrf2 siRNA (Santa Cruz Biotechnology) for 24 h, using DharmaFECT 4 (Thermo Fisher, UK), as per the manufacturer’s recommendations, before treatment of EC with DEM (100 μmol/L) for 24 h.

2.3. Phosphorescence lifetime quantification of O₂ content in endothelial cytosol and medium

Intracellular O₂ levels were measured using a cell-penetrating phosphorescence platinum–porphyrin based nanoparticle probe, MitoXpress®-INTRA (Luxcel Biosciences, Cork, Ireland) [24,25] and a time-resolved fluorescence plate reader (FLUOstar Omega, BMG Labtech, UK) equipped with an atmospheric control unit, allowing for measurement of cytosolic O₂ content under defined O₂ levels. HUVEC were seeded at 50,000 cells/well in 96-well black microtitre plates and loaded after 48 h by incubation with MitoXpress®-INTRA (10 μg mL⁻¹) for 18 h in M199 containing 20% serum.

Oxygen quenches the phosphorescent emission from MitoXpress®-INTRA, such that phosphorescence measured at 360 nm exc and 655 ± 55 nmem is inversely proportional to [O₂]. Phosphorescence intensity was measured at two delay times (30 μs and 70 μs t₇) and converted to phosphorescence lifetime (τ) using the following formula: τ = (t₇−t₈)/ln(f₁/f₂), where f₁ represents the intensity at t₁, τ. Calibration of phosphorescence lifetime to O₂ concentration (%) was achieved by treating cells with the mitochondrial complex III inhibitor antimycin-A (10 μmol L⁻¹), thereby eliminating any O₂ gradient between the medium and cytosol. Stepwise reductions in ambient O₂ levels were performed (21, 18, 13.5, 10, 7.5, 5, 2 and 1% O₂, with glucose (20 mmol L⁻¹)glucose oxidase (50 μg mL⁻¹) used to create 0% O₂ levels) and probe lifetime monitored as described in Fig. S1A and D. Results were plotted against the applied ambient O₂ tension and subjected to exponential fit analysis, with unknown lifetimes subsequently interpolated from this curve. Dissolved O₂ in the medium was measured in parallel by diluting MitoXpress®-INTRA (2.5 μg mL⁻¹) in medium. Detailed validation of MitoXpress®-INTRA for measurement of O₂ content in primary EC, including all calibration functions and cell viability indices are provided in Fig. S1.

2.4. Impedance measurements of cell adhesion and proliferation

HUVEC were cultured in air or adapted to 5% O₂ for 5 days and then seeded at 20,000 cells mL⁻¹ into iCELLigence 8-well E-plates (E8, ACEA Biosciences Inc., USA). Real-time biopimpedance
measurements were obtained using a cell analyzer platform (iCELLigence, ACEA Biosciences Inc., USA) [29] maintained at 37 °C in air or 5% O₂ within the O₂-regulated workstation. Changes in bioimpedance generated by cell coverage over the electrode in each well of the E8 plates was used to assess cell proliferation and expressed as Cell Index (CI), calculated from the difference between cell and background impedance (see Fig. S2C). Proliferation was also assessed by monitoring cell protein content present in 0.5 M NaOH solubilised extracts (see Fig. S2B).

2.5. Measurement of mitochondrial reactive oxygen species generation

Mitochondrial reactive oxygen species generation was measured using the mitochondrial targeted probe MitoSox red, which we have previously shown is inhibited by PEG–SOD and rotenone in HUVEC [28]. HUVEC, cultured in air or adapted for 1 or 5 days to 5% O₂, were loaded in serum-free M199 with MitoSOX red (5 μmol L⁻¹) for 20 min before treatment for 30 min in the absence or presence of 2 μmol L⁻¹ antimycin-A. Fluorescence (Ex 545 nm/Em 602 nm) was detected in 4% paraformaldehyde fixed cells using a Nikon Diaphot microscope, with images captured using an ORCA-03G (Hamamatsu, Japan) camera with 0.89 s exposure. All fluorescence quantification was conducted using uncorrected or contrast images (Fig. S2D). For enhanced print quality, representative cell fluorescence images shown in Fig. S3 were contrast adjusted equally. Corrected total fluorescence units (CTFU) from 3 to 4 fields of view for each condition were quantified by densitometry using Image J, with background and cell area correction applied (Cell integrated density – (cell area*background)).

Fig. 1. Affymetrix microarray identifies differentially expressed diethylmaleate-responsive Nrf2 target genes in human umbilical vein endothelial cells (HUVEC) adapted to physiological O₂ (5%). (A) Affymetrix transcriptome analysis software computed signaling pathways likely to be affected in EC challenged with diethylmaleate (DEM, 100 μmol L⁻¹) for 4 h in air or 5% O₂. Molecules from the data set that met the basal/DEM ratio cut-off of > 1.2 or < −1.2 and P cut-off of < 0.05 were then associated with relevant canonical pathways, with the Nrf2 pathway shown to be the primary pathway induced under both air (hyperoxia) and physiological normoxia (5% O₂) conditions. The volcano plots depict DEM-responsive genes in air (main panel) and 5% O₂ (inset panel) conditions, with the X-axis indicating fold change relative to control and the Y-axis indicating p-value significance of changes in gene expression. Fewer Nrf2-dependent genes were inducible under 5% O₂, with the primary Nrf2 targets GCLM, xCT and HO-1 induced under both conditions. In the inset, HO-1 is denoted in gray, as separate analyses indicated basal and/or DEM responsiveness may be diminished under 5% O₂. Data derived from independent cultures from 5 different donors. Gene Abbreviations: GCLM, glutamate cysteine ligase modifier subunit; SCL7A11/xCT, solute carrier family 7 anionic amino acid transporter light chain; HMOX-1, heme oxygenase-1; NQO1, NAD(P)H quinone oxidoreductase 1; ABC4, multidrug resistance protein member 4; BACH1, BTB and CNC homology 1, basic leucine zipper transcription factor 1; GSR, glutathione reductase; TXNRD1, thioredoxin reductase 1; SQSTM1, sequestosome 1. (B) HUVEC were loaded with the O₂-sensitive, nanoparticle probe MitoXpress®-INTRA to simultaneously measure O₂ content in the culture medium and cytosol (see Fig. S1). Life-time phosphorescence intensity was measured in an O₂-regulated FLUOstar fluorescence plate reader. HUVEC were maintained under the indicated O₂ level for at least 30 min, at which point cytosolic O₂ levels were stable. Dashed lines highlight ambient O₂ levels most commonly used in vitro to mimic in vivo levels, with the shaded box denoting ‘hypoxic’ O₂ levels. (C) Cytosolic O₂ content in HUVEC maintained in air for 60 min before reducing ambient O₂ to 5% (‘physiological normoxia’) and monitoring cytosolic O₂ levels for a further 60 min. Dissolved O₂ in culture medium was monitored in parallel. (D) Summary of cytosolic and medium O₂ content in HUVEC cultured in air or 5% O₂ for 1 or 5 days. Dashed lines represent the ambient O₂ levels measured during culture of EC in air or 5% O₂.
Expression Console software using the RMA algorithm for normalization, background correction and summarization at the gene levels. Principal Component Analysis (PCA) was used (Omics Explore, Qlucore AB, Sweden) to visualize independent HUVEC cultures, providing a multidimensional representation of samples in three dimensions, with each dimension consisting of a signature of probes/variables with a similar pattern across the samples. These components were ranked by the percentage of the variability they represent. Despite donor variability, two groups of samples (control and DEM in EC adapted to air) were clearly separated from each other along the axis of main principal component. Using the Affymetrix Transcriptome Analysis Console, molecules from the data set that met the basal/DEM ratio cutoff of > 1.2 or < –1.2 and P cut-off of < 0.05 (q0.2 air q0.5 5% O2) were used to generate gene lists of DEM-responsive genes and mapped to the relevant canonical pathways.

2.8. Nrf2 binding to ARE using TransAM assay

HUVEC cultured in air or adapted to 5% O2 for 1 or 5 days were treated for 2 h with control medium alone or containing DEM (100 μM) and nuclear proteins extracted (Active Motif) according to the manufacturer’s instructions, as previously described [7]. The binding activity of nuclear Nrf2 to ARE (5′-GTCA-CAGTGACTCACGAGAATCTG-3′) was determined by ELISA (Trans-AM Nrf2 kit, Active Motif).

2.9. Immunoblotting

Whole cell lysates were extracted with SDS lysis buffer (containing protease and phosphatase inhibitor cocktails, pH 6.8) on ice for 10 min [7,28]. Nuclear protein was extracted using a nuclear extraction kit (Active Motif). Denatured samples (10 μg) were separated by gel electrophoresis, electro-transferred onto polyvinylidene difluoride membranes, and then probed with primary and HRP-conjugated secondary antibodies, using α-tubulin and lamin C as reference proteins for whole cell and nuclear protein, respectively [7]. Protein expression was determined by enhanced chemiluminescence with images captured in a gel documentation system (G-BOX, Syngene Ingenius Bioimaging) and densitometry performed using Image J software (National Institute of Health, USA). As specified in the legend to Fig. 3, lanes were spliced to adjust the lane sequence.

2.10. Quantitative RT-PCR (qPCR)

HUVEC were cultured in air or adapted to 5% O2 for 1 or 5 days, treated for 4 h with M199 alone or containing diethylmaleate (DEM, 100 μM) and RNA isolated using a Nucleospin RNA Kit (Macherey-Nagel). A spectrophotometer (NanoDrop, Thermo Scientific, UK) was used to assess sample RNA content and purity. Total RNA was reverse- transcribed using a high capacity cDNA conversion kit (Applied Biosystems) and NQ01, HO-1, GCLM, Nrf2, Keap1 and xCT mRNA assessed by real-time qPCR (Corbett Rotorgene) [7]. mRNA expression was normalized to the geometric mean of β-2-microglobulin (B2M), ribosomal protein L13a (RPL13A) and succinate dehydrogenase unit complex A (SDHA) and primer sequences are listed in Table S1.

2.11. Measurement of intracellular glutathione and ATP levels

HUVEC, cultured in air or adapted for 1 or 5 days to 5% O2, were extracted for glutathione (GSH) with 6.5% trichloroacetic acid on ice for 10 min, and total cellular GSH measured using a fluorometric assay [7]. ATP content was determined using a Firefly luciferase/luciferin reaction mixture for acid extracts of samples or...
ATP standards (0–40 nmol L⁻¹). Bioluminescence was measured for 10 s in a microplate reader (BMG Labtech) [34].

2.12. Statistical analysis

Data denote mean ± S.E.M. of measurements in independent cultures from n = 3–12 different donors. Statistical analyses were conducted using a Student’s t-test or two-way ANOVA followed by Bonferroni’s multiple comparisons, with P < 0.05 considered significant.

3. Results

3.1. Affymetrix mRNA profiling reveals diminished Nrf2 target gene induction in HUVEC adapted to physiological O₂ levels

A preliminary global transcriptome analysis of HUVEC cultured in air or adapted to 5% O₂ for 5 days, revealed that diethylmaleate (DEM, 100 μmol L⁻¹) induced 564 coding transcripts in air, with Nrf2 the pre-dominant pathway activated, including antioxidant (heme oxygenase-1, HO-1) and phase II (glutathione reductase,
thioredoxin reductase-1, NAD(P)H quinone oxidoreductase 1), GSH-related (glutamate-cysteine ligase, Solute Carrier Family 7- anionic amino acid transporter light chain, xCT) and metabolic/ transport/autophagy-related (Multidrug Resistance-Associated Protein 4, Phosphoglucuronate Dehydrogenase, Sequestosome-1) genes (Fig. 1A, main panel). Gene induction at 5% O2 was diminished, with 276 coding transcripts induced by DEM and GCLM and xCT the key targets of DEM challenge (Fig. 1A, inset panel). Comparison of air and 5% O2 basal and DEM responses further indicated that basal and maximal HO-1 induction may be diminished in HUVEC adapted to 5% O2.

3.2. Real-time measurements of medium and intracellular O2 content confirm exposure of cells to physiological O2 levels

Previous studies of the effects of O2 tension on cultured cells have often omitted measurements of the O2 gradient between the medium and intracellular environment, leading to conflicting reports associated with activation of hypoxia-regulated signaling pathways. In the present study, we have monitored real-time cytosolic O2 content in HUVEC exposed to different O2 concentrations (0–15 μg mL⁻¹) and time (6–24 h) dependence of nanoparticle loading in HUVEC cultured in air, confirming that raw phosphorescence values increased but did not translate into differences in lifetime due to the ratiometric conversion for lifetime phosphorescence (Fig. S1A and D). Similarly, intracellular GSH and ATP levels were unaffected in EC loaded for 24 h with the nanoparticle probe MitoXpress™-INTRA. To validate our nanoparticle sensor measurements of intracellular O2 content, we in-

3.3. Effects of physiological O2 on cell adhesion, proliferation and mitochondrial superoxide generation

To characterize the effects of physiological O2 on the phenotype of EC under basal conditions, we monitored adhesion and proliferation of HUVEC during culture in air 5% O2 for 5 days, by measuring cell protein content (Fig. S2B) or using a bioimpedance iCELLigence platform [29] maintained within an O2-regulated workstation (Fig. S2C). Proliferation but not adhesion was reduced during culture under 5% O2, confirming previous observations in primary human venular EC [37] and lymphocytes [19]. To determine the effects of physiological O2 on the redox state of HUVEC, we assayed MitoSox red fluorescence as an index of mitochondrial reactive oxygen species generation. Fluorescence increased transiently after 1 day adaptation to 5% O2, with inhibition of mitochondrial complex III by antimycin A leading to similar increases in superoxide generation in HUVEC cultured in air or 5% O2 for 1 or 5 days (Figs. S2D and S3). The transient increase in superoxide generation is consistent with a previous report of increased dihydroothidixide oxidation in HUVEC exposed acutely (1 h) to 3% O2 [38]. Long-term adaptation of HUVEC to 5% O2 had no effect on protein carbonylation or basal expression of SOD2, catalase or eNOS (data not shown), and transmission electron microscopy confirmed that 5-day adaptation to 5% O2 had negligible effects on cell morphology (see Fig. S2E), with an absence of organelle swelling/distention or loss of parallel double membranes surrounding in the nuclear envelope, endoplasmic reticulum (ER) or mitochondria.

3.4. Physiological O2 has no effect on Nrf2 nuclear translocation although induction of target genes HO-1 and NQO1 is diminished

To further validate whether long-term adaptation to 5% O2 affects Nrf2 signaling, we examined the effects of physiological O2 (5%) on Nrf2 DNA binding capacity and the induction of key downstream targets by real time qPCR and immunoblotting. Activation of Nrf2 by DEM (100 μmol L⁻¹), a classical stimulus known to induce oxidation
of Cys151 on Keap1 [31], was assessed by nuclear Nrf2 accumulation (Fig. 2A) and Nrf2 in vitro DNA binding activity (Fig. 2B). Notably, Nrf2 activation was similar in EC cultured in air or adapted to 5% O₂ for 1 or 5 days. In contrast, protein and mRNA expression of the Nrf2 target genes HO-1 (Fig. 3A and B) and NAD[P]H quinone oxidoreductase 1 (NQO1, Fig. 3C and D) induced by DEM were attenuated in HUVEC adapted to 5% O₂ consistent with a previous report in the murine RAW264.7 macrophage cell line [39]. Notably, attenuation of DEM stimulated HO-1 expression in HUVEC adapted to 5% O₂ was fully reversible on re-adaptation of cells to air for 5 days (Fig. 3E), indicating phenotypic plasticity. To confirm whether attenuated HO-1 induction in HUVEC adapted to 5% O₂ was stimulus specific, we investigated the effects of other known Nrf2 activators. Treatment of HUVEC for 12 h with the lipid peroxidation product 4-hydroxynonenal [40] (Fig. 4A), the isothiocyanate sulforaphane [8] (Fig. 4B) or the nitric oxide donor DETA NONOate [41] (Fig. 4C) led to a marked induction of HO-1 in HUVEC cultured in air, which was significantly attenuated in cells adapted to 5% O₂ for 1 or 5 days. Studies in human coronary artery EC (HCAEC), adapted to 5% O₂ for 5 days and then challenged with 4-hydroxynonenal (12 h, 20 μmol L⁻¹) (Fig. S4), confirmed our findings of attenuated HO-1 and NQO1 induction in primary venous EC.

3.5. Adaptation to physiological O₂ upregulates expression of Bach1, a repressor of HO-1 and NQO1

BTB and CNC homology 1, basic leucine zipper transcription factor 1 (Bach1) lacks transcriptional activity and acts as a negative regulator of HO-1 and NQO1 gene transcription [42] by binding Maf recognition elements [43]. Notably, prolonged hypoxia (1% O₂) has been shown to upregulate Bach1 expression in HUVEC and other cell types [12]. We here report the first evidence that adaptation of HUVEC to physiological O₂ tension (5%) for 1 or 5 days upregulates Bach1 protein (24 h) and mRNA (4 h) expression (Fig. 5A and B) independent of HIF-1α induction (Fig. S2A), with similar findings obtained for Bach1 protein expression in HCAEC (Fig. S4C). siRNA knockdown of Bach1 in HUVEC adapted to 5% O₂ restored diethylmaleate stimulated HO-1 expression (Fig. 5C) to levels observed in EC adapted to air (see Fig. 3).

When we further examined whether adaptation of EC to physiological O₂ affects other modulators of Nrf2 signaling, such as Keap1 (cytosolic binding protein for Nrf2) [2] and DJ-1 (Parkinson disease-associated protein and stabilizer of Nrf2) [44], basal expression of Keap1 and DJ-1 was unaffected following adaptation of HUVEC to 5% O₂ for 1 or 5 days (Fig. S5A and B). Moreover, adaptation of HUVEC to 5% O₂ had negligible effects on NFκB expression (Fig. S5C).

3.6. Physiological O₂ does not affect GSH synthesis despite elevated Bach1 expression

Stress-induced synthesis of the key intracellular thiol GSH is dependent on Nrf2-regulated cystine-glutamate transporter xCT and GCL activity [6,45,46], known to be induced by the electrophilic agent diethylmaleate [30,47]. We here report that basal and diethylmaleate stimulated intracellular GSH levels were

**Fig. 5.** Enhanced Bach1 expression in HUVEC adapted to 5% O₂ underlies decreased induction of HO-1 by diethylmaleate. HUVEC were cultured in air or adapted to 5% O₂ for 1 or 5 days before treatment with M199 or M199 containing DEM (100 μmol L⁻¹) for 4 h or 24 h. (A–B) Representative immunoblot and densitometric analyses of Bach1 protein (24 h) or mRNA (4 h) expression under basal and DEM-stimulated conditions. (C) HUVEC adapted to 5% O₂ for 5 days were transfected with scramble or Bach1 siRNA, and basal and DEM (100 μmol L⁻¹, 24 h) stimulated HO-1 and Bach1 protein expression measured relative to α-tubulin. Data denote mean ± S.E.M., n = 3–7 different donors *P < 0.05, **P < 0.01, ***P < 0.001.
unaffected following adaptation of HUVEC to 5% O₂ for 1 or 5 days (Fig. 6A). Moreover, adaptation to 5% O₂ had negligible effects on basal and diethylmaleate-stimulated expression of the xCT transporter (Fig. 6B) or GCL modulator subunit (GCLM) (Fig. 6C), consistent with our Affymetrix transcriptome data.

In cells cultured in air, Bach1 is reported to repress expression of the Nrf2 targets GCLM and xCT [48]. To assess whether elevated Bach1 levels in EC adapted to 5% O₂ affect GCLM expression, HUVEC were transfected with Bach1 siRNA. Notably, unlike restoration of HO-1 expression following Bach1 knockdown (Fig. 5C), GCLM expression in HUVEC adapted to 5% O₂ was not augmented by knockdown of Bach1 (Fig. 6D), suggesting that under physiological O₂ GSH synthesis is independent of Bach1. As previous studies in other human cell types cultured in air have reported that GCLM is negatively regulated by Bach1 [48], we conducted further 'control' experiments in HUVEC cultured in air. As shown in Fig. S6, knockdown of Bach1 in HUVEC cultured in air enhances GCLM expression, confirming previous reports in other human cell types [48]. Collectively, these results suggest that GSH-related genes may mediate transducers of Nrf2-regulated antioxidant defenses under physiological O₂ due to their insensitivity to Bach1 regulation.

4. Discussion

Our study establishes that the O₂ environment in which endothelial cells are cultured in vitro significantly influences inducible but not basal redox defenses regulated by Nrf2. Our findings reveal that adaptation to physiological O₂ (5%) significantly alters the regulation of select Nrf2 targets genes by Bach1. We provide the first evidence that Bach1 is upregulated in human primary endothelial cells adapted long-term to physiological O₂ in the absence of HIF-1α stabilization. Moreover, elevated Bach1 levels regulate a smaller subset of Nrf2 defense genes than those previously identified under standard atmospheric culture conditions [48]. Although the induction of HO-1 and NQO1 is diminished in endothelial cells adapted to 5% O₂, notably GSH levels and expression of GCLM and xCT are unaffected, suggesting that under physiological O₂ levels encountered in vivo genes related to GSH synthesis may mediate Nrf2-regulated cellular protection.

The O₂ concentration in the umbilical vein in vivo approximates 3.7% [35] and, as blood flow minimizes the formation of significant O₂ gradients, cellular O₂ levels should mimic this level closely. In previous studies, primary HUVEC were adapted long-term to 3% O₂ in an attempt to mimic O₂ levels in vivo [36] but, as illustrated in Fig. 1B, this results in a cytosolic O₂ content of ~2% and an hypoxic phenotype characterized by enhanced HIF-1α and target gene expression [36]. Moreover, use of O₂-regulated workstations and plate readers has enabled us to study cells under defined O₂ tension, obviating oxidative stress induced by reoxygenation during routine cell culture. We employed an O₂-sensitive nanoparticle probe to demonstrate that O₂ content in EC decreases as dissolved O₂ in the culture medium is lowered from 18% to 5% O₂, and confirmed an intracellular O₂ content of 3.6% in HUVEC adapted long-term to 5% O₂. Although upregulation of HIF-1α in EC exposed to hypoxia decreases Nrf2-driven luciferase and...
HO-1 expression [49], the cytosolic O$_2$ level (3.6%) achieved in our experiments after 5 days adaptation of HUVEC to 5% O$_2$ did not increase HIF-1$\alpha$ protein levels (Fig. S 2A).

Previous studies have shown that changes in O$_2$ tension can affect the phenotype of cells, resulting from alterations in the activity of ion channels, kinases and redox sensitive genes [14–18]. Despite these reports, our observations concerning basal redox status following long-term adaptation of primary HUVEC to 5% O$_2$ revealed no effect on protein carbonylation, basal expression of SOD2, catalase or eNOS (data not shown). Furthermore, our EM data revealed negligible changes in cell morphology (Fig. S2E) following long-term adaptation.

Diminished Nrf2 regulated HO-1 induction under 5% O$_2$ was agonist independent (Fig. 4) and reversible upon re-adaptation to air, indicating plasticity of redox defenses in primary human venous [Fig. 3] and arterial [Fig. S4] EC adapted to different O$_2$ tensions. Our experiments suggest that upregulation of Bach1 in primary endothelial cells under physiological O$_2$ levels may account for the downregulation of HO-1 and NQO1 induction [12,50] rather than reduced nuclear Nrf2 translocation (Fig. 2). This hypothesis is further supported by our finding that knockdown of Bach1 in endothelial cells adapted to 5% O$_2$ restores diethylmaleate stimulated HO-1 induction. Increased heme levels are known to inhibit DNA binding of Bach1, enabling transcriptional activators to bind to HO-1 enhancers [42,51,52]. Bach1 has been reported to regulate HO-1 mRNA and/or protein expression (HUVEC, HCAEC or ECV304) under hypoxia (1% O$_2$) represses HO-1 mRNA expression without affecting mRNA degradation [55].

Elevated Bach1 expression in human primary endothelial cells adapted to physiological O$_2$ (5%) inhibits Nrf2-regulated transcription of HO-1 and NQO1. Notably, long-term adaptation of cells to 5% O$_2$ does not impair the upregulation of glutathione related genes GCLM and xCT.

**Fig. 7.** Differential regulation of Nrf2-targeted genes in human endothelial cells adapted to physiological O$_2$ levels encountered in vivo. Elevated Bach1 expression in human primary endothelial cells adapted to physiological O$_2$ (5%) inhibits Nrf2-regulated transcription of HO-1 and NQO1. Notably, long-term adaptation of cells to 5% O$_2$ does not impair the upregulation of glutathione related genes GCLM and xCT.
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Supplementary Information

**Bach1 differentially regulates distinct Nrf2-dependent genes in human venous and coronary artery endothelial cells adapted to physiological oxygen levels**

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Fig. S1. Validation of MitoXpress®-INTRA for measurements of medium and cytosolic \( \text{O}_2 \) content in intact HUVEC. (A) HUVEC were loaded with MitoXpress®-INTRA (5, 10 and 15\( \mu \text{g.mL}^{-1} \)) for 6, 16 or 24 h under room air, and phosphorescence intensity measured (left panel) and converted into phosphorescence lifetime (\( \mu \text{s}, \) right panel), as described in Methods. Raw phosphorescence measurements show loading time-and concentration-dependent increases which did not translate into differences in lifetime due to the ratiometric conversion for lifetime phosphorescence. (B and C) HUVEC cultured in ambient air or adapted to 5% \( \text{O}_2 \) (normoxia) for 5 days were loaded with MitoXpress®-INTRA (5, 10 or 15\( \mu \text{g.mL}^{-1} \)) for 24 h, and intracellular GSH and ATP levels measured. (D) HUVEC were loaded with 10\( \mu \text{g.mL}^{-1} \) MitoXpress®-INTRA for 18 h in air and then treated with 10\( \mu \text{M} \) antimycin A to inhibit mitochondrial respiration. Cells were exposed to stepwise reductions in ambient \( \text{O}_2 \) in an \( \text{O}_2 \)-regulated fluorescence plate reader, whilst continuously monitoring phosphorescence lifetime (\( \mu \text{s} \)) to obtain real-time changes in intracellular \( \text{O}_2 \). MitoXpress®-INTRA dissolved in medium in the absence of cells provides a measure of medium \( \text{O}_2 \) content. Mean phosphorescence lifetime (\( \mu \text{s} \)) values were plotted against the applied ambient \( \text{O}_2 \) concentration and subjected to an exponential fit analysis to generate a calibration function (inset). This was subsequently used to convert phosphorescence lifetime into \( \text{O}_2 \) (%). Data are representative of independent cultures from 3 different donors.
**Fig. S2.** HIF-1α levels, cell adhesion, proliferation and mitochondrial ROS generation in human umbilical vein endothelial cells (HUVEC) adapted to physiological oxygen (5% O₂). (A) Representative immunoblot and densitometric analysis of basal HIF-1α protein expression relative to α-tubulin in HUVEC cultured in air or adapted to 5% O₂ or 1% O₂ for 5 days. (B) HUVEC were seeded at 20,000 cells/well and protein content measured over 1 to 4 days. (C) Proliferation of HUVEC assessed over 4 days by real-time cell bioimpedance using an iCelligence platform, with data expressed as cell index. (D) Mitochondrial superoxide levels measured using MitoSOX red in the absence or presence of the mitochondrial complex III inhibitor antimycin-A (2μM, 30min) in HUVEC cultured in air or 5% O₂ for 1 or 5 days. Data denote mean ± S.E.M., n = 3-7 different donors, *P<0.05, **P<0.01, ***P<0.001 vs. 1 day adapted EC and ##P<0.01, ###P<0.001, ####P<0.0001 versus corresponding condition in 5% O₂ unless indicated otherwise. (E) Transmission electron micrograph of HUVEC cultured in air or adapted to 5% O₂ for 5 days. Monolayers were fixed in 2.5% glutaraldehyde/0.1M cacodylate buffer for 4 h at room for subsequent electron microscopy. Arrows denote particular organelles or morphological features discernible. **Abbreviations:** M, mitochondria; N, nucleus; Weibel-Palade bodies, WP; NM, nuclear membrane; ER, endoplasmic reticulum; G, Golgi; P, phagocytic vacuoles.
Fig. S3. Representative MitoSOX red fluorescence in HUVEC cultured in air or adapted to 5% O₂. Mitochondrial superoxide levels were measured using MitoSOX red in the absence or presence of the mitochondrial complex III inhibitor antimycin-A (2µM, 30min) in HUVEC cultured in air or adapted to 5% O₂ for 1 or 5 days.
Fig. S4. Adaptation of human coronary artery endothelial cells to physiological O\textsubscript{2} levels alters HO-1, NQO1 and Bach1 protein expression. Primary HCAEC were cultured in air or adapted to 5% O\textsubscript{2} for 5 days and then treated for 12h with 4-hydroxynonenol (HNE, 20\textmu mol.L\textsuperscript{-1}) or vehicle (Veh, 0.01% hexane). Representative immunoblots and densitometric analysis of HO-1 (A), NQO1 (B) and Bach1 (C) expression relative to \( \alpha \)-tubulin. Data denote mean ± S.E.M., \( n = 4 \) independent HCAEC cultures, *\( P<0.05 \), **\( P<0.01 \), ***\( P<0.001 \).
**Fig. S5.** Keap1, DJ-1 and NFκB p65 protein expression in HUVEC cultured in air or adapted long-term to different O\textsubscript{2} levels. (A-B) HUVEC were cultured in air or adapted to 5% O\textsubscript{2} for 1 or 5 days and then treated for 24 h with medium alone or containing the electrophile diethylmaleate (DEM, 100µmol.L\textsuperscript{-1}) and lysates immunoblotted for Keap1 and DJ-1. (C) Comparison of basal NFκB protein expression in HUVEC cultured in air or adapted to 5% O\textsubscript{2} or 1% O\textsubscript{2} for 5 days. Densitometric analysis of Keap1, DJ-1 and NFκB (p65) expression was relative to α-tubulin. Data denote mean ± S.E.M., n = 3-8 independent cultures from different donors.
**Fig. S6.** Bach1 siRNA increases GCLM protein expression in HUVEC cultured in air. HUVEC were transfected with scramble (Scr) or Bach1 siRNA, and basal and diethylmaleate (DEM, 100µmol.L⁻¹, 24 h) stimulated GCLM expression measured relative to α-tubulin. Data denote mean ± S.E.M., n = 4 different donors, *P<0.05, **P<0.01.
Table S1. Primer sequences for qPCR measurements in human umbilical vein endothelial cells

<table>
<thead>
<tr>
<th>Gene names</th>
<th>Sequence (5’-3’)</th>
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<tbody>
<tr>
<td><strong>HO-1</strong></td>
<td>Sense AGGCCAAGACTGCGTTCCCT</td>
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<tr>
<td>NQO1</td>
<td>Sense GCCCAGATATTTGCTGGCTGA</td>
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<tr>
<td>xCT</td>
<td>Sense GGGAAAGTCTTGGAACCTCAGG</td>
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<td>Bach1</td>
<td>Sense GCAGCAGTTCCACTCAAG</td>
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<tr>
<td>Nrf2</td>
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<td><strong>Reference genes</strong></td>
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<td>SDHA</td>
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<tr>
<td>RPL13A</td>
<td>Sense GAGGCCCCCTACCACCTCC</td>
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