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Oxygen deprivation inhibits basal keratinocyte proliferation in a model of human skin and induces regio-specific changes in the distribution of epidermal adherens junction proteins, aquaporin-3, and glycogen

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Abstract

It is generally accepted that hypoxia and recovery from oxygen deprivation contribute to the breakdown and ulceration of human skin. The effects of these stresses on proliferation, differentiation and expression of cell-cell adhesion molecules were investigated for the first time in an organotypic model of human skin. Fully stratified tissues were exposed to a time course of oxygen deprivation and subsequent reoxygenation. Regional changes in keratinocyte morphology, glycogen stores and cellular junctions were observed, with more differentiated layers of the epidermis exhibiting the first evidence of oxygen deprivation. Cellular swelling within the granular layer was concurrent with aquaporin-3 depletion.

The keratinocyte adherens junction proteins E-cadherin and β -catenin were dramatically decreased in a regio-specific manner throughout the epidermis following oxygen deprivation. In contrast, P-cadherin and the desmosomal proteins desmoplakin and desmoglein-1 were refractory to oxygen deprivation. Relative to normoxic controls, hypoxic tissues exhibited increased mRNA levels of the transcriptional repressor Slug however mRNA levels of the related transcriptional factor Snail were unaffected. All cellular and molecular changes were reversible upon reoxygenation. These results demonstrate that oxygen deprivation and reoxygenation exert differential effects on epidermal adhesion proteins and suggest a novel role for cadherins, β -catenin, and Slug in hypoxia-induced junctional changes occurring in stratified squamous epithelium.

Keywords

Hypoxia; Epidermis; Keratinocyte; Chronic Wound

Introduction

The response of normal human stratified squamous epithelium to oxygen deprivation and the molecular and morphological pattern of recovery from this stress are largely unknown. While skin is considered mildly-to-moderately hypoxic under steady-state conditions,^{1–5} impairment of regional blood vessels by disease or wounding exacerbates the hypoxic status of this organ.

It is well established that chronic levels of hypoxia, caused by obstructed blood flow over long periods of time, hamper proper tissue regeneration and promote infection.^{6,7} The ability of human skin to resist damage following oxygen depletion and/or reoxygenation is clinically relevant to both acute and chronic wounds.

Cycles of regional oxygen deprivation and reoxygenation in human skin are believed to contribute to loss of tissue integrity, ultimately resulting in ulceration. Cell-cell adhesion via structures such as desmosomes and adherens junctions is responsible for maintaining the overall tensile strength of the human epidermis.⁸ However, little is known about the effects of decreased oxygen availability on the intercellular junctions of human skin. Loss of tissue integrity negatively impacts the ability of wounded epithelium to heal. In ischemic carcinomas a loss of polarity and cell-cell adhesion is due in part to degradation of the essential transmembrane portion of the adherens junction protein, E-cadherin. P-cadherin is specifically located to the basal and immediately suprabasal layers of the human epidermis, although its response to hypoxia and its function in wounded or stressed human skin have not been previously investigated.

Snail and Slug are well known regulators of epithelial-mesenchymal transition in the developing embryo.^{9,10} The presence of Slug within basal keratinocytes of the mouse epidermis and at the wound edges of monolayer human keratinocyte cultures suggests its involvement in the re-epithelialization stages of wound healing.^{11,12} Microarray analysis of the epidermis of Slug-null mice has provided additional evidence for Slug's role in keratinocyte differentiation, as well as proliferation, apoptosis, adhesion and motility.¹³ Furthermore, Slug has been shown to contribute to the migratory and invasive phenotypes of numerous carcinoma cell lines through down-regulation of E-cadherin expression.^{11,14–17} These recent reports point to a putative role for Slug as a transcriptional regulator involved in maintenance of human skin integrity through effects on epidermal cadherin protein expression.

Since the epidermis is distanced from the dermal circulation and variations in blood flow are common, keratinocyte carbohydrate stores are critical for maintaining this renewal tissue. The human epidermis has a high metabolic rate, particularly in comparison to the dermis,¹⁸ and employs anaerobic glycolysis as its main form of cellular metabolism.¹⁹ Early studies showed that actively dividing cells, such as those found in the basal layer of the human epidermis, rapidly utilize glycogen stores for energy.²⁰ Epidermal glycogen levels are dramatically increased following tissue perturbation such as wound healing, ultraviolet irradiation, psoriasis or the removal of the stratum corneum by tape-stripping.^{21–24} There are no reports on the effects of hypoxic stress on epidermal glycogen storage and basal keratinocyte proliferation in human skin.

Here we use a bioengineered model of human skin to investigate the effects of oxygen deprivation on epithelial tissue independent of glucose or nutrient levels. The NIKS human keratinocyte cell line, first described in 2000, is spontaneously long-lived with a near-diploid karyotype that remains stable over continuous passaging.²⁵ NIKS keratinocytes exhibit growth requirements and differentiation patterns that are identical to primary keratinocytes, they possess wild-type p53 alleles and are non-tumorigenic in athymic nude or severe combined immunodeficiency mice. When combined with a dermal compartment consisting of human dermal fibroblasts and type I collagen, NIKS keratinocytes form a three-dimensional tissue containing both epithelial and connective tissues. Gene array analysis shows that gene expression in organotypic cultures generated from NIKS and primary human keratinocytes exhibit a high level of concordance. Thus, they provide an important model for the study of stratified epithelium's responses to compromised oxygen levels and recovery from hypoxia. While striking alterations in keratinocyte morphology, aquaporin-3, specific adherens junction proteins, glycogen storage patterns and cellular proliferation were all observed during oxygen

deprivation, these tissue responses were reversible upon reoxygenation. In the epidermis, the levels and distribution of the adherens junction proteins E-cadherin and β -catenin were altered following oxygen deprivation whereas P-cadherin and desmosomal proteins were unaffected. Loss of E-cadherin and β -catenin, as well as concomitant induction of Slug mRNA, was an early response of human skin to hypoxia. Our studies reveal dynamic molecular and morphological tissue responses to oxygen status which may contribute to acute and chronic cutaneous wound healing, as well as susceptibility to ulcer formation.

Materials and methods

Organotypic culture

Monolayer cultures of human NIKS keratinocytes or primary keratinocytes were grown using mitomycin-C treated Swiss mouse 3T3 fibroblast feeder layers as previously described.²⁵ The dermal component of the organotypic cultures and culturing media were provided by Stratatech Corporation (Madison, WI). Dermises were lifted to the air-liquid interface before plating of keratinocytes.²⁶ Triplicate tissues were grown for each experiment. Monolayer cultures and tissues were maintained at 37°C in a humidified 5% CO₂ atmosphere.

Microarray Analysis

Triplicate organotypic cultures created from primary keratinocytes and NIKS were harvested on day 15 using 1ml of TRI Reagent from a RiboPure™ Kit (Applied Biosystems/Ambion, Austin, TX). Gene expression profiling and analysis were performed by GenUs Biosystems (Northbrook, Illinois) using CodeLink Whole Human Genome microarray (Applied Microarrays, Tempe, AZ). The data generated for each probe on the array platform were analyzed with GeneSpring GX v7.3.1 software (Agilent Technologies, Santa Clara, CA). To compare individual expression values across the array, raw intensity data from each gene was normalized to the median intensity of the array. Only genes with values greater than background intensity in at least one treatment condition were used for further analysis. Using a ratio interpretation of the data and normalization of each gene to the median intensity across conditions, data was filtered by expression intensity for genes that did not vary by 50% across all samples within the experiment. Only genes with $p \leq 0.05$ (Student's t-test) between groups were used to find differentially expressed genes with at least 2-fold changes across groups.

Hypoxia and reoxygenation treatments

Organotypic cultures of NIKS human keratinocytes were grown a minimum of 12 days under normoxic (5% CO₂, balance air) conditions at 37°C. Samples were then exposed to hypoxia by sealing them in Modular Incubator Chambers (Billups-Rothenberg, Delmar, CA) flushed with 5% CO₂ / 95% N₂ for 10 minutes. Tissues were maintained for 2, 4, 8, 12, 24 or 48 hours at 37°C until harvested at day 14 post-plating. For reoxygenation experiments, cultures were similarly cultured under normoxic conditions for 12 days, sealed in incubator chambers for 24 hours at 37°C and restored to normoxic conditions for 24, 48, 72 or 96 hours. For all experiments, tissues were fed with non-degassed media prior to hypoxic treatments. Parallel tissues were maintained for identical time periods under normoxia as controls.

Histology preparation

Tissues were fixed in 1% paraformaldehyde and divided evenly for further cryopreservation and paraffin-embedding. All paraffin-embedding and tissue sectioning was performed by the Histology Laboratory (Department of Pathology, University of Wisconsin-Madison, Madison, Wisconsin). Paraffin-embedded sections (5 μ m) were stained with hematoxylin and eosin (H&E) for evaluation of tissue morphology and periodic-acid Schiff (PAS) to assess glycogen storage. Adjacent sections were treated with diastase to control for PAS staining of

mucopolysaccharides or collagen. Stained sections were viewed using an Olympus IX-70 microscope (Center Valley, PA). Digital images were captured by an Optronics DEI-750 CE camera (Goleta, CA) using ImagePro Plus software (Media Cybernetics, Silver Spring, MD).

Immunoblotting

Cell lysate was collected from individual organotypic cultures using 200 μ l CytoBuster lysis buffer (Novagen, EMD Biosciences, Darmstadt, Germany) and Protease Inhibitor Cocktail #3 (Calbiochem, EMD Biosciences, Darmstadt, Germany). Protein concentrations were determined by BCA assay (Pierce Chemical Co., Rockford, IL), 20 μ g total protein was separated on a 4–12% Bis-Tris NuPAGE gel in MOPS buffer (Invitrogen, Carlsbad, CA) and transferred to a 0.45 micron Immobilon P PVDF membrane (Millipore, Bedford, MA). Blots were probed with affinity-purified polyclonal goat anti-human HIF-1 α antibody (1:2,000) (R&D Systems, Minneapolis, MN), followed by donkey anti-goat IgG horseradish peroxidase (1:25,000) (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were stripped by incubation with 62.5 mM Tris (pH 6.8), 2% SDS and 100 mM β -mercaptoethanol at 50°C for 30 min for re-probing with mouse anti-human β -actin (Sigma, Saint Louis, MO) and goat anti-mouse IgG (Roche, Indianapolis, IN). Blots were developed using ECL Advance Chemiluminescence detection reagent per manufacturer's instructions (Amersham, Piscataway, NJ) and imaged using Kodak Image Station IS2000R (New Haven, CT).

Indirect immunofluorescence analysis

For E-cadherin, β -catenin and caspase-3 staining, paraffin-embedded sections (5 μ m) were deparaffinized with xylenes, rehydrated through an ethanol series, incubated 5 minutes in 1% SDS in PBS at room temperature for antigen exposure, washed in PBS and blocked with 3–10% normal goat serum (NGS) (Sigma, St. Louis, MO). For filaggrin staining, sections were similarly deparaffinized, rehydrated and then boiled in 10 mM citrate buffer (pH 6.0) for 10 minutes, cooled and blocked in 5% NGS. For P-cadherin, aquaporin-3, desmoglein-1, desmoplakin and Ki67 staining, cryopreserved tissue sections (5 μ m) were fixed in cold acetone and blocked with 3% NGS in PBS. The following antibodies and dilutions were used: anti-E-cadherin (1:80), anti-P-cadherin (1:20), anti- β -catenin (1:50), anti-desmoglein-1 (1:50) (BD Pharmingen, San Diego, CA), anti-caspase-3 (1:50) (R&D Systems, Minneapolis, MN), anti-filaggrin (1:100) (NeoMarkers, Inc., Fremont, CA), anti-rat aquaporin-3 (>85% sequence homology with human, 1:500) (Sigma, St. Louis, MO), anti-desmoplakin (1:50) (Abcam, Cambridge, MA), and anti-Ki67 (1:20) (Oncogene, Boston, MA). Tissue sections were incubated with primary antibody for 1 hour at 37°C (anti-E-cadherin incubated 3 hours at 37°C) and secondary antibodies (1:500, goat anti-rabbit IgG-Alexa 488 or goat anti-mouse IgG-Alexa 488, Molecular Probes, Eugene, OR) for 30 minutes at room temperature. Nuclei were stained with 5 μ g/ml Hoechst 33258 in PBS. Stained tissue sections were examined and digital images captured as described above. Dual color images were created by overlaying single color digital images taken of the same field.

Quantification of cellular proliferation and apoptosis

Indirect immunofluorescent staining for Ki67 and caspase-3 was performed on tissues from three individual experiments from each hypoxic and reoxygenation time point. For quantification of proliferative cells, the number of Ki67-positive basal cells within 6 random microscope fields was manually counted. For quantification of apoptotic cells, the number of caspase-3-positive cells found within the length of the entire epidermal tissue section was manually counted. Results from triplicate experiments were averaged for both cellular stains. Statistical significance of the differences observed between experimental groups was determined by one-way ANOVA using GraphPad Prism software (San Diego, CA).

Comparisons between group means were made with the Bonferroni test for multiple comparisons.

Quantitative PCR

VEGF, aquaporin-3, E-cadherin, Snail and Slug mRNA levels were measured using quantitative PCR analysis of triplicate wells from three independent experiments. Total RNA was isolated from tissues in 1 ml TRIzol Reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Following DNase I treatment of 2 µg total RNA using the DNAfree kit (Ambion, Austin, TX), reverse transcription was performed using oligo dT primers and M-MLV reverse transcriptase per manufacturer's instructions (Invitrogen). Quantitative PCR was performed using the Chromo4 Four-Color Real-Time PCR Detector (Bio-Rad, Hercules, CA) with the following human primer sets in conjunction with SYBR Green Supermix (Bio-Rad): VEGF 5'-GAGTACATCTTCAAGCCATCC-3' (forward) and 5'-TGAGGTTTGATCCGCA TAATC-3' (reverse); aquaporin-3 5'-AGATGCTCCACATCCGCTAC-3' (forward) and 5'-ATGAGGATGCCAGAGTGAC-3' (reverse); E-cadherin 5'-CCGCCATCGCTTACACATCC-3' (forward) and 5'-CTCTCTCGGTCCAGCCAGTG-3' (reverse); Snail 5'-ACCGCTCGCTGCCAATG-3' (forward) and 5'-AGCCTTCCCACTGTCCTCATC-3' (reverse); Slug 5'-GACCCTGGTTTGCTTCAAGGA-3' (forward) and 5'-TGTTGCAGTGAGGGCAAGAA-3' (reverse); Cyclophilin A 5'-CAAGGTCCCAAAGACAGCAGAA -3' (forward) and 5'-CACCTGACACATAAACCTG-3' (reverse). All mRNA levels were normalized to human cyclophilin expression. Values were compared to matched normoxic control cultures from each time point, which were arbitrarily set to 1. Opticon Monitor Software and the Genex Excel Macro (Bio-Rad) were used for data analysis. Statistical significance of the differences observed between experimental groups was determined by one-way ANOVA using GraphPad Prism software. Comparisons between group means were made with the Bonferroni test for multiple comparisons.

Results

NIKS organotypic cultures express hypoxia- and cell junction-related genes in the same manner as primary keratinocytes

To ensure that the NIKS keratinocytes would provide an accurate model system in which to study hypoxia and related conditions, gene array analysis was performed comparing the gene expression profiles of organotypic cultures generated from either NIKS or primary keratinocytes. Differences in expression (increases or decreases) between NIKS and primary keratinocytes of two-fold or greater were considered significant. Concordance between organotypic cultures generated with NIKS or primary human keratinocytes was greater than 97% for 113 genes associated with hypoxia responsiveness and angiogenesis. Furthermore, in the evaluation of 36 genes related to glycogen synthesis, no alterations in gene expression were observed. We next examined a subset of specific genes associated with keratinocyte differentiation, signaling, development and physiology. These were identified as genes of interest in the examination of exposure of the NIKS organotypic cultures to hypoxic conditions. When gene expression of individual genes was compared, no differences between NIKS and primary keratinocytes were found (Table 1).

Exposure of human stratified squamous epithelium to hypoxia increases hypoxia-related mRNA and proteins

To investigate the tissue-specific response to hypoxia in our model of human skin, RNA and protein lysates were harvested from stratified tissues exposed to a hypoxic time course that extended from two to 48 hours. The oxygen-responsive transcription factor HIF-1α and the HIF-1α-regulated downstream gene, vascular endothelial growth factor (VEGF), were

measured to confirm that a hypoxic state was induced in our tissue model. HIF-1 α protein levels reached maximal levels in whole tissues after 12 hours of hypoxic exposure (Figure 1a). As expected, VEGF mRNA levels increased linearly with extended hypoxic exposure (Figure 1b). The level of VEGF mRNA present in tissues exposed to greater than eight hours of hypoxia was statistically different from the level present after two hours of exposure to an oxygen-free atmosphere.

Hypoxia reversibly affects cell size and enucleation events in organotypic cultures of human keratinocytes

Since the majority of studies on hypoxia have used malignant cell lines or mouse models,^{2–4,27} the organotypic cultures of human keratinocytes used in our studies provided a unique opportunity to temporally analyze hypoxic responses within the context of a three-dimensional, fully differentiated, intact human organ. The morphological effects of hypoxia and subsequent reoxygenation on stratified epithelium were visualized with hematoxylin and eosin (H&E) staining and compared to normoxic controls (Figure 2a). Throughout the hypoxic time course, no obvious changes in the connective tissues of the dermis were observed at the light microscope level. However, a linear progression toward a hypoxic cellular phenotype within the epidermis was observed with the most dramatic alterations noted after 24 and 48 hours of oxygen deprivation (Figure 2b–d). An obvious displacement of nuclei was observed in a subset of cells within all living layers of the epidermis. Nuclear material was shifted to the perimeter of the nucleus, resulting in vacuoles. The granular layer underwent striking morphological changes over time following hypoxia. After 24 hours of hypoxia, cells within this layer exhibited hydropic swelling and had lost their intracellular granules (black bars, Figure 2b–d). The observed nuclear phenotype and increase in cell size were evident in keratinocytes of the upper suprabasal layers and progressed to cells in an immediate suprabasal position over time. Few basal keratinocytes exhibited nuclear changes or an increase in cell size. However, after 24 to 48 hours of hypoxia, intercellular spaces were observed between adjacent keratinocytes within the basal layer. Despite dramatic alterations in cellular morphology at the extended hypoxic time points, tissues remained structurally intact after 48 hours of hypoxia. None of the tissues analyzed showed visual evidence of intra-epidermal or epidermal-dermal separation.

Epithelial tissues were examined histologically for their ability to recover from the observed morphological changes upon reoxygenation. Tissues that had been exposed to 24 hours of hypoxia were allowed to recover for 24 to 96 hours under normoxic conditions (reoxygenation). Cellular vacuoles and swelling appeared exacerbated after 24 hours of reoxygenation (Figure 2e), however, the stratified epithelium was able to recover from the dramatic hypoxic phenotype within 48 hours of reoxygenation (Figure 2f). Relative to normoxic controls, morphological features of the epidermis, such as keratinocyte cell size, nuclear shifting and vacuoles as well as the presence of granules, were restored at the 48 hour time point. Of note, a dense layer of flattened, nucleated keratinocytes located immediately superior to the granular layer was present at all time points following reoxygenation (black brackets, Figure 2e–h). This layer was presumed to be the remnant of the swollen keratinocytes of the granular layer induced during hypoxia. After 72 to 96 hours of reoxygenation the tissue morphology of hypoxic stratified epithelial tissues did not differ from normoxic controls (Figure 2g, h), with the exception of the defined parakeratotic layer in the innermost region of the stratum corneum.

To further investigate the cellular swelling of keratinocytes within the granular layer observed during the hypoxic time course, levels of aquaporin-3, the water and glycerol transporter found on the surface of keratinocytes, were measured. With increasing time in hypoxia, decreases in aquaporin-3 mRNA levels were observed (Figure 2i). Indirect immunofluorescent staining revealed that aquaporin-3 protein was specifically localized to the cell membrane, most prominently in the suprabasal layers under normoxic conditions (Figure 2j). Hypoxic tissues

exhibited a decrease in cell membrane-associated aquaporin-3 protein, particularly within the granular layer (Figure 2k). Cytoplasmic localization of aquaporin-3 protein was also apparent in the uppermost differentiated layers. Levels of filaggrin protein, which localizes to keratohyalin granules within the granular layer, were also found to decrease after 48 hours of hypoxia (Figure 2l, m).

Hypoxia causes metabolic stress on stratified epithelial layers, altering localization of glycogen stores

It has been suggested that glycogen may serve as a primary energy source for skin exposed to prolonged ischemia.²⁸ Periodic acid-Schiff (PAS) staining for glycogen was used to determine whether the stress of hypoxia and reoxygenation affected the glycogen distribution pattern in our model of normal human skin. As expected, intact foreskin and adult breast tissue lacked PAS staining within the interfollicular epidermis (data not shown). Under normoxic conditions, glycogen stores were located within keratinocytes comprising the spinous and granular layers of cultured tissues, indicated by dark purple staining (Figure 3a). No glycogen stores were present in basal keratinocytes, presumably due to the high metabolic rate of cells in this proliferative layer. PAS staining observed in the stratum corneum was attributed to non-specific staining, due to similar patterns observed in control tissue sections treated with the enzyme diastase (Figure 3h).

Tissues exposed to hypoxic stress for up to 12 hours had no glycogen stores present in any living stratified layers (Figure 3b). Conversely, increased levels of PAS staining were observed at 24 and 48 hours after exposure to hypoxia, with localization patterns that differed dramatically from normoxic controls. After 24 hours of oxygen deprivation, high levels of glycogen staining were present in keratinocytes located in the basal and spinous layers (Figure 3c), while staining was mainly localized to basal keratinocytes at the 48 hour time point (Figure 3d). Nuclear glycogen staining was present within the vacuoles created by the displacement of keratinocyte nuclei. Staining was notably absent in the more differentiated, swollen cells of the granular layer of hypoxic tissues, in contrast to the distribution observed in normoxic control tissues.

Similar to the 24 hour hypoxic time point, PAS staining was present in all living layers of the stratified epidermis after 24 and 48 hours of reoxygenation (Figure 3e, f). After 72 hours of reoxygenation, glycogen storage patterns resembled normoxic controls. Staining was localized to the suprabasal layers at both 72 (Figure 3g) and 96 hour (data not shown) reoxygenation time points, indicating the epithelium required a longer period to reestablish a normal pattern of glycogen distribution within the tissue than to recover its morphological appearance (compare with Figures 2e–h).

Reduction of basal keratinocyte proliferation during hypoxia and acute reoxygenation is reversible, with no increase in apoptosis

The effect of hypoxia and reoxygenation events on keratinocyte proliferation was measured using indirect immunofluorescent staining for the nuclear antigen Ki67. For all samples, Ki67-positive cells were found only in the basal layer; no suprabasal keratinocyte proliferation was observed. Compared to normoxic controls, decreases in cellular proliferation were observed after tissues were exposed to hypoxia for 48 hours (Figure 4a). A statistically significant reduction in the number of Ki67-positive basal keratinocytes was also observed in tissues reoxygenated for 24 hours following 24 hours of hypoxia. The number of proliferative cells present in tissues reoxygenated for 48 hours was comparable to normoxic tissues, indicating that the effect on proliferation was transitory and the proliferative compartment of the tissue remained replication-competent (Figure 4b). Additionally, tissues exposed to 24 and 48 hours of hypoxia did not express increased levels of the apoptotic protein caspase-3 (Figure 4c).

Decreases in E-cadherin-containing adherens junction proteins during hypoxia are reversed upon reoxygenation

Indirect immunofluorescent staining was used to visualize the expression of the transmembrane adherens junction proteins E-cadherin and P-cadherin, and the intracellular signal transduction molecule β -catenin. Protein staining patterns of hypoxic and reoxygenated tissues were compared to normoxic controls. As expected, under normoxic culturing conditions E-cadherin protein was appropriately localized to the cell membrane of the basal, spinous and granular layers of the stratified tissue (Figure 5a). However, E-cadherin protein levels were markedly decreased by 24 hours of hypoxic exposure (Figure 5b). After 48 hours of hypoxia, minimal E-cadherin protein was detected (Figure 5c). Protein loss occurred in a time-dependent manner and was first evident in the upper, more differentiated layers. After 48 hours of hypoxia only slight amounts of E-cadherin protein were observed in the basal layer and protein expression was specifically localized to the basolateral surface of the basal keratinocytes. Within 24 hours of reoxygenation, expression of E-cadherin protein was restored to the cell surface of all living stratified layers (Figure 5d). In contrast to E-cadherin, P-cadherin protein expression was not affected by oxygen deprivation. Under normoxic conditions, P-cadherin expression was localized exclusively to the basal and immediately suprabasal layers (Figure 5i), consistent with its localization in intact human skin. P-cadherin protein expression levels and localization were not affected after 24 or 48 hours of hypoxia (Figure 5j, k). Tissue reoxygenation also had no effect on this basally-located adherens junction protein (Figure 5l).

A third adherens junction protein, the intracellular signal transduction molecule β -catenin, was also investigated. Similar to E-cadherin, β -catenin protein was localized to the cell membrane under normoxic conditions (Figure 5e) and was markedly decreased by hypoxia (Figure 5f, g). In contrast to E-cadherin, β -catenin protein was maintained at the cell membrane in basal keratinocytes, with punctate expression at the basolateral surface of cells within the basal layer (Figure 5g, inset). β -catenin protein losses were localized to the more differentiated layers, with modest changes observed in the basal keratinocytes. Loss of β -catenin protein expression in the suprabasal layers was also reversible within 24 hours of reoxygenation, with restoration of localization to the cell membrane (Figure 5h). Recovery of both E-cadherin and β -catenin protein expression was maintained throughout all remaining reoxygenation time points, up to 96 hours (data not shown).

Desmosomal-specific proteins are largely unaffected by hypoxic exposure

The desmosomal proteins desmoplakin and desmoglein-1 were visualized by indirect immunofluorescence to determine if these cell-cell junctions were also altered by hypoxia and reoxygenation. Both desmosomal proteins were present throughout all living stratified layers of the normoxic tissue (Figure 6a, e). Exposure to hypoxia did not affect desmoplakin protein levels or localization in the stratified tissues, even after 24 and 48 hours (Figure 6b, c). Differentiated tissue layers exhibited a slight increase in cytoplasmic staining for desmoplakin protein as the length of hypoxic exposure increased, but overall protein levels remained comparable to normoxic tissue samples. Desmoplakin protein levels in tissues re-introduced to normal oxygen levels were unaltered (Figure 6d).

Desmoglein-1 protein exhibited elevated expression levels in the uppermost granular layer and at the basal surface of the basal keratinocyte layer (Figure 6e). Lengthened hypoxic exposures did not affect overall desmoglein-1 protein levels in the lower portion of the tissue; however, the dense staining pattern observed immediately below the stratum corneum was diminished after 48 hours of hypoxia (Figure 6f, g). This extended period of oxygen deprivation also resulted in a modest shift from cytoplasmic to membrane-bound protein localization in this uppermost region. Desmoglein-1 protein expression after 24 hours of reoxygenation was identical to normoxic controls (Figure 6h).

Slug mRNA levels are increased in stratified squamous epithelium exposed to hypoxia

To investigate the mechanism responsible for decreased E-cadherin protein levels observed during hypoxia, mRNA levels of the E-cadherin transcriptional repressors Snail and Slug were measured. Quantitative PCR was used to measure Snail and Slug mRNA levels in stratified tissues exposed to hypoxia and reoxygenation. After 24 and 48 hours of hypoxia, Slug mRNA levels were increased approximately 6-fold in comparison to normoxic control tissues (Figure 7a). Conversely, Snail levels increased only slightly, not exceeding a 2-fold increase over normoxic controls at the 24 and 48 hour time points. The increase in mRNA from the Slug transcriptional repressor correlates with the decrease in E-cadherin protein levels observed at these time points. Upon 24 hours of reoxygenation, tissue E-cadherin mRNA levels were more than 10-fold greater than hypoxic tissues that were not exposed to reoxygenation (Figure 7b). Both Snail and Slug mRNA levels returned to pre-treatment levels.

Discussion

The cell type-specific cellular and molecular responses of intact human skin to compromised oxygen levels are presently understood at a rudimentary level. Both tissue regeneration (wound healing) and tissue degeneration (cutaneous ulceration) occur in an environment of decreased oxygen availability. Most studies employ monolayer culture methods to investigate cellular responses to hypoxic stress.^{14,29,30} Utilizing a model of human skin that includes both epidermal and dermal compartments to investigate the tissue's response to decreased oxygen availability provides a level of biological relevance not possible in monolayer culture of individual cell types. This study uses NIKS organotypic cultures which have been demonstrated to express genes associated with hypoxia at a similar level to organotypic cultures generated from primary keratinocytes. The oxygen deprivation system employed by this study enabled us to segregate the epidermal response to oxygen levels, independent of nutrient availability, thereby eliminating this aspect of the ischemic response. Equally important, we show the remarkably resilient nature of normal human epidermal tissue and its ability to recover from short-term hypoxia.

In this study, we show that diminished oxygen availability causes profound and reversible morphological and metabolic alterations in the human stratified epithelium. Hypoxia elicited regio-specific modifications in aquaporin-3 levels, cellular adhesion proteins and glycogen storage that were initiated in the more differentiated layers of the tissue. The hydropic swelling observed in our model of human skin may be associated with aberrant water transport within the epidermal tissue. Indeed, a dramatic reduction in the main water transporting molecule, aquaporin-3, were observed. Decreases were found at both the mRNA and protein level. Diminished aquaporin-3 levels are also associated with decreased cellular migration and proliferation in epidermal wounds.³¹

While the adherens junction proteins E-cadherin and β -catenin were decreased and/or redistributed by hypoxia, we show here for the first time that desmosomal junction and basally-located P-cadherin proteins were unaffected. These data indicate that desmosomal and P-cadherin-containing adherens junctions are critical for maintaining epidermal architectural integrity during hypoxic events in human skin. Consistent with this interpretation, no intra-epidermal or epidermal-dermal separation or degradation was found. We further propose that Slug, a transcription factor involved in E-cadherin regulation, plays a role in the response of human keratinocytes to hypoxia. Implication of this well-characterized transcription factor in the biological response of keratinocytes to hypoxia provides a possible point of therapeutic intervention to forestall the effects of reperfusion injury. Furthermore, based on our findings, P-cadherin appears to be regulated in a HIF-1 α - and Slug-independent manner.

Defining the role of the transcription factors Snail and Slug in the response of normal stratified epithelium to hypoxia may help define the mechanisms involved in oxygen-dependent changes in cell-cell adhesion. Few studies focus on Snail or Slug's regulation of cellular adhesion in non-malignant or unwounded tissues. We observed an upregulation of Slug mRNA, without an increase in Snail mRNA, in response to hypoxia in our model of human interfollicular epidermis. Similarly, Savagner *et al.* report the presence of Slug mRNA in cells at the migrating wound edge of monolayer HaCaT keratinocyte cultures, while Snail mRNA expression was not affected.³² Additionally, Turner *et al.* report that activation of Slug down-regulated expression of E-cadherin and integrin molecules, as well as decreased cellular proliferation, in studies using primary human keratinocytes.³³ Although both studies used human keratinocytes, their experiments were performed in monolayer culture under normoxic conditions, precluding analysis of hypoxia-induced effects on Snail or Slug. Use of an organotypic model of stratified human epithelium in the work presented here helps define these roles within the context of a dimensional tissue. Our results provide further evidence of the importance of the transcriptional repressor Slug in controlling expression of the adherens junction protein E-cadherin in the epithelial cell type.

The differential responsiveness of E- and P-cadherin to hypoxia was an unexpected and novel finding. This is the first report to show hypoxia is capable of differentially affecting two closely-related cadherin molecules. The maintenance of cell surface P-cadherin on basal keratinocytes throughout the hypoxic time course may contribute to the cell type-specific renewal properties of the interfollicular epidermis. A proliferative stem cell compartment of human epidermis resides within the basal layer, where P-cadherin protein is localized. This area is also believed to be mildly hypoxic in normal skin.^{2,3} The specificity of hypoxia's effect on E-cadherin, but not P-cadherin, may serve to protect the keratinocyte progenitor cells within the replicative basal layer from acute, and perhaps chronic, oxygen deprivation. Additionally, the specificity of hypoxia-mediated down-regulation of E-cadherin, while not affecting P-cadherin protein expression, highlights the reciprocal relationship these two adherens junction proteins share. Our findings point to the potentially important role P-cadherin-containing adherens junctions and desmosomes play in maintaining the integrity of epidermis.

In adherens junctions the intracellular portion of cadherins binds to β -catenin. β -catenin is a potent nuclear signaling molecule, tightly regulated through phosphorylation, and plays an important role in differentiation of human skin.²⁶ In our skin model, hypoxia induced a loss of β -catenin from the cell membrane of suprabasal but not basal keratinocytes. We propose that hypoxia-induced changes in regio-specific β -catenin localization may play a role in adaptation of the differing layers of epidermis to acute changes in oxygen levels. In support of this interpretation, it has been recently shown that under hypoxic conditions β -catenin is found directly bound to the HIF-1 transcription factor in the nucleus.³⁴ In these studies Kaidi and coworkers demonstrated that HIF-1 α / β -catenin binding resulted in decreased cellular proliferation of colorectal cancer cells. Conversely, resolution of the hypoxic stress resulted in loss of HIF-1 α / β -catenin binding and concomitant restoration of proliferation. Similar to Kaidi *et al.*, cellular proliferation was restored in our model of human skin following reoxygenation. These findings warrant additional mechanistic studies and illustrate the importance of tissue-engineered models for the investigation of tissue-specific pathophysiology induced by oxygen deprivation.

Hypoxia caused regio-specific changes in epidermal glycogen content in our model. Lobitz and Holyoke hypothesized that glycogen storage is inversely correlated with mitotic cell activity, based on results from early tape-stripping experiments performed on human epithelium.²¹ Indeed, staining of our tissues for the proliferative nuclear antigen Ki67 indicated cellular proliferation was decreased after 48 hours of hypoxia while PAS staining revealed glycogen was present in basal keratinocytes at this time point. These stores were also present

during earlier time points of tissue reoxygenation, indicating cellular proliferation had not returned to control levels. This was further supported by the significant reduction in Ki67 staining at the earliest recovery time point of 24 hours. Restoration of normal glycogen metabolism, as well as proliferation, was observed by 48 to 72 hours following reoxygenation. This biphasic glycogen utilization pattern, with initial depletions followed by increased glycogen content, was also reported by Lobitz and Holyoke and is likely indicative of tissue-specific adaptation to a limited oxygen environment.²¹ These results illustrate the temporal and biphasic response of epidermal cellular metabolism to local oxygen levels.

In conclusion, using a model of human skin, we have identified novel tissue-specific responses to acute hypoxia and reoxygenation. The system presented here fulfills an unmet need for epidermal organ models capable of mimicking the *in vivo* state of tissue damage during hypoxia and reoxygenation. Recently, skin harvested from ischemic, amputated limbs has been used to investigate the role of dermal tissue in epidermal ulceration.^{35,36} Studies such as these highlight the fact that models of chronically hypoxic or ischemic skin are essential to our understanding of problematic cutaneous wound healing.³⁷ Skin organ models are powerful tools not only for identification of dynamic molecular and morphological tissue responses to oxygen status but also for investigation of epithelial-stromal interactions which ultimately contribute to ulcer formation.

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List of abbreviations

HIF-1 α , Hypoxia-inducible factor-1 α ; VEGF, vascular endothelial growth factor; PAS, Periodic acid-Schiff.

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Figure 1. Exposure of stratified human epithelium to hypoxia causes increased levels of the oxygen-responsive transcription factor HIF-1 α and a HIF-1 α target gene

(a) Immunoblotting for HIF-1 α performed on protein lysates from stratified epithelial tissues (top; 120 kD) showed maximal protein expression after 12 hours of hypoxia. β -actin (bottom; 42 kD) served as an internal loading control. (b) Quantitative PCR was performed on cDNA from triplicate stratified epithelial tissues using VEGF-specific primers. Fold expression was expressed compared to time-matched normoxic controls and all samples were normalized to human cyclophilin A expression. Error bars represent standard error of the mean ($n = 3$). * $p < 0.01$, compared with 2 hour hypoxic time point.

Figure 2. The normal human epidermis undergoes reversible morphological changes upon exposure to hypoxia, with increased cell size and alterations in the granular layer
Hematoxylin and eosin staining of tissue sections (a) cultured under normoxic conditions; exposed to hypoxia for (b) 12 hours, (c) 24 hours or (d) 48 hours; reoxygenated following 24 hours of hypoxia for (e) 24 hours, (f) 48 hours, (g) 72 hours and (h) 96 hours. Black bars indicate swollen granular cells, brackets indicate condensed supragranular layer. Representative images from three individual experiments are shown. (i) Quantitative PCR analysis of mRNA from tissues exposed to hypoxia using primers specific for aquaporin-3. Fold expression was expressed compared to time-matched normoxic controls. Error bars indicate standard error of the mean (n = 3). Indirect immunofluorescent staining for aquaporin-3 (green) from (j) normoxic and (k) 48 hour hypoxic tissues and filaggrin (green) from (l) normoxic and (m) 48 hour hypoxic tissues. Nuclei were counterstained with Hoechst 33258 (blue). Staining was performed on sections from three individual experiments, representative images shown. Scale bars = 50 μm.

Figure 3. The stress of hypoxic exposure alters glycogen storage in the normal stratified epithelium which is reversible upon reoxygenation

PAS staining (purple) of tissue sections (a) cultured under normoxic conditions; exposed to hypoxia for (b) 12 hours, (c) 24 hours or (d) 48 hours; reoxygenated following 24 hours of hypoxia for (e) 24 hours, (f) 48 hours and (g) 72 hours. (h) Diastase control for non-glycogen staining. Staining was performed on sections from three individual experiments, representative images shown. Scale bar = 50 μ m.

Figure 4. Hypoxic exposure decreases cellular proliferation with no increase in apoptosis (a, b) Cellular proliferation (Ki67) and (c) apoptosis (caspase-3) were measured using indirect immunofluorescent staining. Tissues from three individual experiments were stained from each hypoxic and reoxygenation time point indicated. Positive basal cells within 6 random microscope fields (Ki67) or within the entire tissue section length (caspase-3) were manually counted and all values were averaged. No Ki67 staining was observed in suprabasal keratinocytes. Error bars represent standard error of the mean. * $p < 0.05$, ** $p < 0.01$.

Figure 5. E-cadherin-containing adherens junction proteins are reversibly down-regulated by hypoxic exposure of stratified human epithelium

Indirect immunofluorescent staining for the adherens junction proteins E-cadherin (a-d, green), β -catenin (e-h, green) and P-cadherin (i-l, green) was performed on stratified epithelial tissues. Tissues were cultured under normoxic conditions (a, e, i) or exposed to hypoxia for 24 (b, f, j) or 48 (c, g, k) hours. Samples reoxygenated for 24 hours (d, h, l) were also stained. Nuclei were counterstained with Hoechst 33258 (blue). Staining was performed on sections from three individual experiments, representative images shown. Scale bar = 50 μ m.

Figure 6. Desmosomal proteins are largely unaffected by exposure of stratified epithelium to hypoxia

Indirect immunofluorescent staining for the desmosomal proteins desmoplakin (a–d) and desmoglein-1 (e–h) was performed on stratified epithelial tissues. Tissues were cultured under normoxic conditions (a, e) or exposed to hypoxia for 24 (b, f) or 48 (c, g) hours. Samples reoxygenated for 24 hours (d, h) were also stained. Staining was performed on sections from three individual experiments, representative images shown. Scale bar = 50 μ m.

Figure 7. Slug mRNA levels are elevated after prolonged hypoxic exposure

Quantitative PCR for E-cadherin, Snail and Slug was performed on cDNA from stratified epithelial tissues exposed to the hypoxic (a) and reoxygenation (b) time points indicated. Hypoxic samples were compared with matched normoxic control cultures for each hypoxic time point (a). Reoxygenated samples were compared to hypoxic tissues that did not undergo reoxygenation (b). All samples were normalized to human cyclophilin A. Representative data from one of three independent experiments is shown. Error bars represent standard deviation. * $p < 0.001$, compared to 2 hour time point.

Table 1

Individual Gene Expression Comparison Between NIKS and Primary Keratinocytes

Gene	Fold expression (NIKS / Primary)
E-cadherin	0.82
P-cadherin	1.12
β -catenin	1.47
Aquaporin-3	1.75
Desmoplakin	1.17
Desmoglein-1	1.29
Snail	0.88
Slug	1.71