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Original Article

Effects of different hypoxia degrees on endothelial cell cultures—Time course study

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ABSTRACT

Introduction: Exposure of the endothelial cells to hypoxia, the decrease in oxygen supply can trigger an endothelial response. This response is involved in inflammatory diseases, tumorigenesis, and also with the micro vascular damage associated with aging.

The aim of our study was to determine the hypoxia/re-oxygenation induced response *in vitro*, using human umbilical vein endothelial cells (HUVEC) cultures, at different time points with focus on cell viability, apoptosis oxidative stress and angiogenesis stimulation.

Materials and methods: Cells were exposed to 10%, 5% or 0% O_2 for 6 h, 12 h, and 24 h. Viability was measured through colorimetry, apoptosis – annexin V-FITC staining, DNA lesions (γH_2AX), endothelial activation (sICAM1), angiogenesis (HIF1α), oxidative stress (malondialdehyde, superoxidismutase and NFκB activation) were determined by ELISA, Western Blot and spectrophotometry.

Results and discussion: Hypoxia decreased viability, increased apoptosis, oxidative stress, endothelial activation and angiogenesis, depending on O_2 concentration and time exposure. Short exposures to 5% and 10% O_2, efficiently activated anti-apoptotic mechanisms through NFκB activation, HIF1α and γH_2AX related DNA damage repair pathways. However, severe hypoxia and longer exposures to mild hypoxia induced high oxidative stress related damage and eventually led to apoptosis, through strong increases of HIF1α and accumulating DNA lesions.

1. Introduction

The inner layer of the blood vessels wall, the endothelium, is an active tissue, with important surface properties that function as a barrier for circulating macromolecules and it also reacts to a variety of stimuli from the microenvironment (Bouletreau et al., 2002). Exposure of the endothelial cells to hypoxia, the decrease in oxygen supply (Anwar et al., 2014) can trigger an endothelial response. This response is involved in inflammatory diseases, tumorigenesis, and also with the micro vascular damage associated with aging (Wu et al., 2014). Studies on carotid bodies from old and young rats showed that chronic hypoxia and aging lead to the same alterations, such as reduction in volume and density of mitochondria and that there may be a link between aging and hypoxia (Di Giulio et al., 2003).

The endothelial cells can be activated during inflammation or ischemia/reperfusion conditions, which trigger the secretion of numerous molecules, or may eventually lead to cell death through necrosis and/or apoptosis (Zhao et al., 2003). Hypoxic stimulation of the endothelial cells was involved in higher oxidative stress formation through generation of reactive oxygen species (ROS), along with decreased antioxidant capacity (Anwar et al., 2014). The underlining mechanism seems to be represented by nuclear transcription factor kappa B (NFκB) activation (Stempien-Otero et al., 1999). Hypoxia also increased hypoxia inducible factor 1α (HIF1α) expression leading to an angiogenic and apoptotic response (Anwar et al., 2014; Kumar et al., 2011). The overexpression of HIF-1α was responsible for the induction

Abbreviations: Bax, Bcl-2-associated X protein; CHOP, C/EBP-homologous protein 10; DNA, dezoxiribonucleic acid; FITC, fluorescein isothiocyanate; GAPDH, (Glyceraldehyde 3-phosphate dehydrogenase; GRP78, glucose-regulated protein 78; HIF1α, hypoxia inducible factor 1α; HUVEC, human umbilical vein endothelial cells; iNOS, inducible nitric oxide synthetase; IκBα, inhibitor of kappa B; MDA, malondialdehyde; NFκB, nuclear transcription factor kappa B; NOX4, NADPH Oxidase 4; Rac1, ras-related C3 botulinum toxin substrate 1; ROS, reactive oxygen species; SA-β-Gal, senescence-associated beta-galactosidase; sICAM1, soluble intercellular adhesion molecule-1; SOD, superoxide dismutase; VEGF, vascular endothelial growth factor; γH2AX, histone variant H2AX phosphorylated on Ser 139

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of autophagy leading to reduced human umbilical vein endothelial cells (HUVEC) viability (Wu et al., 2014). Moreover, the pattern of apoptosis in the HUVECs exposed to hypoxia was different compared to other cell types. Apoptosis occurred at 12 h of hypoxia and was decreased at 36 h, afterwards the level of apoptosis was again increased at 48 h, 60 h and 72 h of hypoxia. This pattern seemed to be consistent with the thioredoxin expression and the variation of the Bax (Bcl-2-associated X protein)/Bcl-2 ratio in HUVEC that was lower at 6 h and 48 h of hypoxia (Park et al., 2010). However, the underlining mechanism of the endothelial response to hypoxia and its role in pathology is not fully understood.

Therefore the aim of our study was to determine the hypoxia/reoxygenation induced response *in vitro*, using HUVEC cultures, at different time points with focus on cell viability, apoptosis oxidative stress and angiogenic stimulation.

2. Materials and methods

2.1. Cell source

Human umbilical vein endothelial cells (HUVEC, European Collection of Authenticated Cell Cultures ECCAC Porton Down, Salisbury, UK) were used. The cells were grown in RPMI medium supplemented with 5% fetal calf serum, 50 µg/ml gentamycin and 5 ng/ml amphotericin (Biochrom Ag, Berlin, Germany). Cell cultures in the 23rd to 28th passages were used.

2.2. Hypoxia exposure

Growth medium was replaced with fresh medium equilibrated with hypoxic gas mixture, and cells were placed in the incubator (Salvis Vacucenter, version 92/1, Rotkreuz, Switzerland) to a gas mixture of 5% CO_2, and 10%, 5% or 0% O_2 and respectively 85%, 90% or 95% N_2, humidified with water vapors, at 37 °C for 6 h, 12 h, and 24 h. The gases composition was measured using a gas chromatograph (Shimadzu GC-2010, Tokyo, Japan) and the gas flow was continuously monitored using a flowmeter (Krohne, Duisburg, Germany). Cells were then allowed to re-oxygenate for 2 h in standard cell culture conditions.

2.3. Viability testing

Cells cultured at a density of 10^4/well on ELISA 96 wells culture plates (TPP, Switzerland) were settled for 24 h, then exposed to hypoxia, (as described above, Section 2.2.) Viability was measured by colorimetric measurement of a coloured compound – formazan, generated by viable cells using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega Corporation, Madison, USA). All experiments were performed in triplicate. Untreated cultures were used as controls (Baldea et al., 2013).

2.4. Fluorescence microscopy

Cells were seeded in chamber slides at a density of 5×10^4/chamber, allowed to settle 24 h then exposed to hypoxia. Treated cells were stained with Annexin V-fluorescein isothiocyanate (FITC) (BD Pharmingen Biosciences, San Jose, CA, USA) according to the manufacturer's instructions and then fixed in 2% paraformaldehyde. Viable cells were Annexin V negative, apoptotic cells were identified as Annexin V-FITC positive (green) cells. Apoptotic cells were scored by eye, using a fluorescent microscope Olympus BX41 equipped with an Olympus E330 camera driven by software Olympus Master version 1.41EX (USA), at a magnification of 40 × and at least 200 cells per condition were examined.

2.5. ELISA

Soluble intercellular adhesion molecule-1, (sICAM1) ELISA Immunoassay kit from R&D Systems, Inc (Minneapolis, USA) was used. Cells supernatants were treated according to manufacturer's instructions; readings were done at 450 nm with correction wavelength set at 540 nm, using an ELISA plate reader (Tecan, Austria).

2.6. Cell lysis

The cell lysates used in the following experiments were prepared as previously described (Baldea et al., 2015). Protein concentrations were determined by the Bradford method according to the manufacturer's specifications (Biorad, Hercules, California, USA) and using bovine serum albumin as standard. For all assays the lysates were corrected by total protein concentration.

2.7. Western blotting

Lysates (20 µg protein/lane) were separated by electrophoresis on SDS PAGE gels and transferred to polyvinylidenedifluoride membranes, using Biorad Miniprotean system (BioRad). Blots were blocked and then incubated with antibodies against: NFκB, phospho- pNFκB p65 (Ser536) (93H1) (pNFκB), (Cell Signaling Technology, Inc, Danvers, USA), HIF1α, (Santa Cruz Biotechnology, Delaware Ave, Santa Cruz, USA), γH_2AX (histone variant H2AX phospho-Ser 139) (Stressgen Bioreagents Corporation,Victoria, BC, Canada) then further washed and incubated with corresponding secondary peroxidase-linked antibodies (Santa Cruz Biotechnology). Proteins were detected using Supersignal West Femto Chemiluminiscent substrate (Thermo Fisher Scientific, Rockford IL, USA), and a Gel Doc Imaging system equipped with a XRS camera and Quantity One analysis software (Biorad). GAPDH (Glyceraldehyde 3-phosphate dehydrogenase, Trevigen Biotechnology Gaithersburg, MD, Maryland, USA) was used as a protein loading control (Baldea et al., 2013). HUVEC cultures samples exposed to hypoxia for 6 and 24 h were analyzed by Western Blot.

2.8. Oxidative stress-induced damage

To investigate the hypoxia induced oxidative stress in HUVEC cultures, quantification of malondialdehyde (MDA) a marker for the peroxidation of membrane lipids (Ayala et al., 2014) and superoxide dismutase enzymatic activity (SOD) were performed by spectrophotometry, as previously described. All reactives were purchased from Sigma. Data were expressed as nM/mg protein and respectively Units/mg protein (Baldea et al., 2015, 2013).

2.9. Statistical method

The statistical difference between treated and control groups were evaluated by unpaired Student TTEST, two-way ANOVA followed by Bonferroni posttest using GraphPad; results were considered significant for $p \leq 0.05$. Statistical package used for data analysis was Prism version 4.00 for Windows, GraphPad Software, San Diego, California, USA, www.graphpad.com (Baldea et al., 2013; Olteanu et al., 2015; Tudor et al., 2017)

3. Results

3.1. Cell viability

Viability was increased when cells were exposed to mild hypoxia (10% O_2) in a time dependent manner. Exposure to 5% O_2 had initially a stimulating effect, seen by increased viability at 6 and 12 h, then, viability decreased at 24 h. In the absence of oxygen, HUVEC cultures showed decreased viability depending on time exposure (Fig. 1).