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# Effects of helium on inflammatory and oxidative stress-induced endothelial cell damage

Kirsten F. Smit [a], Raphaela P Kerindongo [a], Anita Böing [b], Rienk Nieuwland [b], Markus W. Hollmann [a], Benedikt Preckel [a], Nina C. Weber [a,*]

[a] Academic Medical Centre (AMC), Department of Anesthesiology, Laboratory of Experimental Intensive Care and Anesthesiology (L.E.I.C.A.), Academic Medical Centre (AMC), University of Amsterdam, Meibergdreef 9, 1100 DD Amsterdam, The Netherlands
[b] Department of Experimental Clinical Chemistry, Academic Medical Centre (AMC), Meibergdreef 9, 1100 DD Amsterdam, The Netherlands

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ABSTRACT

Helium induces preconditioning in human endothelium protecting against postischemic endothelial dysfunction. Circulating endothelial microparticles are markers of endothelial dysfunction derived in response to injury. Another noble gas, xenon, protected human umbilical vein endothelial cells (HUVEC) against inflammatory stress in vitro. We hypothesised that helium protects the endothelium in vitro against inflammatory and oxidative stress. HUVEC were isolated from fresh umbilical cords and grown upon confluence. Cells were subjected to starving medium for 12 h before the experiment and treated for either $3 \times 5$ min or $1 \times 30$ min with helium (5% $CO_2$, 25% $O_2$, 70% He) or control gas (5% $CO_2$, 25% $O_2$, 70% $N_2$) in a specialised gas chamber. Subsequently, cells were stimulated with TNF-α (40 ng/ml for 24 h or 10 ng/ml for 2 h) or $H_2O_2$ (500 μM for 2 h) or left untreated. Adhesion molecule expression was analysed using real-time quantitative polymerase chain reaction. Caspase-3 expression and viability of the cells was measured by flowcytometry. Microparticles were investigated by nanoparticle tracking analysis. Helium had no effect on adhesion molecule expression after TNF-α stimulation but in combination with oxidative stress decreased cell viability ($68.9 \pm 1.3\%$ and $58 \pm 1.9\%$) compared to control. Helium further increased TNF-α induced release of caspase-3 containing particles compared to TNF-α alone ($6.4 \times 10^6 \pm 1.1 \times 10^6$ and $2.9 \times 10^6 \pm 0.7 \times 10^6$, respectively). Prolonged exposure of helium increased microparticle formation ($2.4 \times 10^9 \pm 0.5 \times 10^9$) compared to control ($1.7 \times 10^9 \pm 0.2 \times 10^9$).

Summarized, helium increases inflammatory and oxidative stress-induced endothelial damage and is thus not biologically inert. A possible noxious effects on the cellular level causing alterations in microparticle formation both in number and content should be acknowledged.



## 1. Introduction

Ischemic preconditioning results in protection of organs against ischemia/reperfusion (I/R) injury by short, non-lethal periods of ischemia [1]. Besides ischemia, inhalation of volatile anesthetics [2] and noble gases [3] can induce preconditioning. The noble gas helium, which is already routinely and safely used in hospitals worldwide for asthma treatment, has no relevant hemodynamic and neurocognitive side effects, and could be the perfect preconditioning agent for future clinical applications. We recently demonstrated that helium induces both early and late preconditioning in human endothelium in vivo and attenuates postischemic endothelial dysfunction following 20 min of forearm I/R in healthy volunteers [3]. Decreased expression of the pro-inflammatory marker CD11b and intracellular adhesion molecule 1 (ICAM-1) on leucocytes[4] after helium treatment in human volunteers has been reported.

In a former study we could show that intermitted treatment with the noble gas xenon decreased ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1) expression after stimulation with TNF-α in human umbilical vein endothelial cells (HUVEC), thereby protecting endothelial cells against TNF-α induced damage [5].

These data seem to be of special importance since the endothelium plays a major role during I/R and serves as a first line defence mechanism against organ and tissue injury. The protective functions of the endothelium include anti-coagulation, anti-inflammation, prevention of platelet function and regulation of permeability and vascular tone. During I/R, interactions between endothelial cells and blood constituents result in recruitment of

* Corresponding author. Fax: +31 20 6979441.
*E-mail address:* N.C.Hauck@amc.uva.nl (N.C. Weber).

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circulating leucocytes at inflammation sites. These processes are regulated by cytoskeleton alterations and expression of cell adhesion molecules such as ICAM-1, VCAM-1, and E-selectin [6]. The release of pro-inflammatory cytokines such as TNF-α accelerate this process. In myocardial injury, reperfusion after ischemia leads to formation of reactive oxygen species that contribute to the damage inflicted.[7] Increased levels of reactive oxygen species may lead to apoptosis. Apoptosis of endothelial cells precede that of myocardial cells following I/R of the heart, and is assumed to be mediated by caspase-3 release.[8] Circulating microparticles in plasma are a marker of endothelial damage. These particles are derived from endothelial cells after injury and are used as a quantitative marker of endothelial cell dysfunction in patients [9, 10]. Interestingly, exposure to high pressure noble gases, including helium, caused oxidative stress-induced microparticles production in neutrophils [11].

We investigated two different protocols, the first consisting of short, repetitive stimuli (3 × 5 min) helium administration and the second consisting of one prolonged stimulus of 30 min helium administration.

We here hypothesized that pre-treatment with helium protects HUVEC against inflammatory and oxidative stress-induced damage and decreases adhesion molecules, caspase-3 expression, and endothelial cell-derived microparticles, and increases cell viability after I/R.

## 2. Material and methods

All experiments were performed in a specialised temperature controlled gas chamber (Fig. 1). Gas mixtures were administered via standard procedure as described before [5], and outlet gas concentrations were monitored by a gas analyzer (Capnomatic Ultima, Datex, Helsinki, Finland). We used a mixture of helium (5% $CO_2$, 25% $O_2$, 70% helium) and a mixture of control gas (5% $CO_2$, 25% $O_2$, 70% $N_2$) both provided by Linde Gas Benelux, Schiedam, the Netherlands).

### 2.1. Materials

If not mentioned otherwise, all materials used were from Sigma (Zwijndrecht, the Netherlands). Endothelial cell growth medium was obtained from Promocell (Heidelberg, Germany), medium M199 from PAN biotech (Aidenbach, Germany), Fetal Bovine Serum (FBS) from PAA (Colbe, Germany), Penicillin-Streptomycin, Amphotericin B, Trypsine-EDTA from Gibco (Paisley, UK), L-glutamine 200 mM from Gibco (Paisley, UK), and collagenase A from Roche (Mannheim, Germany). AnnexinV-Fluorescein isothiocyanate (FITC) and IgGpoly-FITC were obtained from Immuno Quality Products (Groningen, The Netherlands), anti-human Caspase 3 monoclonal antibody from BD Pharmingen, Franklin Lakes, NJ) and sheep anti human von Willebrand factor-FITC from Serotec (Wiesbaden, Germany).

### 2.2. Isolation of human umbilical vein endothelial cells (HUVEC)

HUVEC were collected from human umbilical veins as described previously [5] (Waiver: W12-167#12.17.096, Ethical Committee AMC, Amsterdam). Cells were cultured in gelatine (0.75%) coated 25- $cm^2$ flasks (passage 0). Experiments were performed with cells of passage 3 and 4. HUVEC were identified using antibodies against von Willebrand factor. Fluorescence-activated cell sorting analysis (FACS) of von Willebrand factor revealed that the cell preparation was 99.6% pure (data not shown). All experiments were performed 3 times.

### 2.3. Experimental protocol

The experimental protocol is outlined in Fig. 1. Upon confluence, cells were put in a resting medium (M199, containing 100 mM L-glutamine) supplemented with penicillin-streptomycin, amphotericin B, and extra L-glutamine (200 mM) for 10 h. After each cycle the medium was refreshed to assure complete washout of the treatment. The short pre-treatment protocol consisted of 3 × 5 min of gas (3 L/min) subsequently followed by 3 × 5 min of rest medium. The long pre-treatment consisted of 30 min of gas treatment (3 L/min) without interruptions. After the respective pre-treatment protocol, HUVEC were either stimulated with $H_2O_2$ (500 μM for 2 h), TNF-α (10ng/ml for 2 h for analysis of adhesion molecules, and 40 ng/ml for 24 h for viability) or left untreated.

### 2.4. Flowcytometry analysis

Attached cells were removed using trypsine and subsequently neutralized with M199 supplemented with FBS10%. Detached cells were isolated from culture supernatants by centrifugation (218g, 4 °C for 10 min). Both cell suspensions were separately centrifuged (218g, 4 °C for 10 min) and resuspended with PBS supplemented with 1% FBS. Adherent cells were resuspended with 600 μl PBS/FCS1%, and detached cells were resuspended in 300 μl PBS/FCS1%. Cell suspensions of both, attached and detached cells, and supernatant were prepared for analysis of caspase 3 and annexin V as previously described [12]. For analysis of caspase 3 we used the FITC active caspase 3 apoptosis kit from BD biosciences (San Diego, CA, USA). Both, attached and detached cell suspensions were prepared for analysis as previously described [12]. Samples were analysed in a fluorescence automated cell sorter (FACS Calibur)

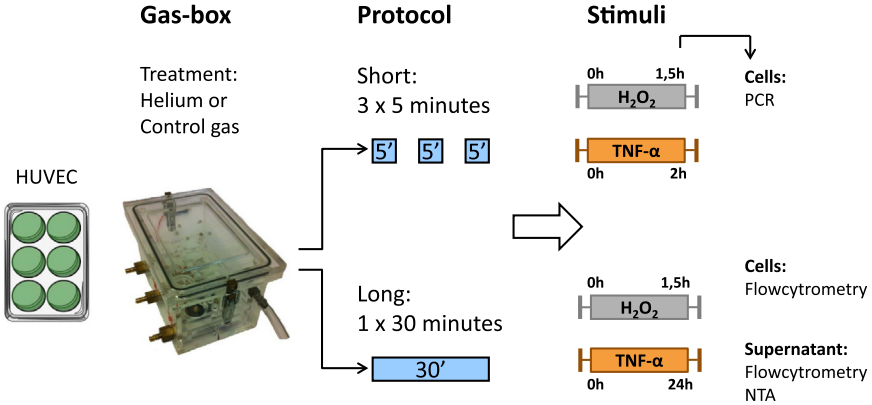


**Fig. 1.** Protocol Outline. The short protocol consisted of 3 times 5 min helium (70%), after each cycle, media were exchanged to ensure washout. Long protocol consisted of one cycle of 30 min helium after which medium was exchanged.