Biochimica et Biophysica Acta 1810 (2011) 526–531

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Limited DNA damage in human endothelial cells after hyperbaric oxygen treatment and protection from subsequent hydrogen peroxide exposure

J. Yuan [a], R.D. Handy [a,*], A.J. Moody [a], G. Smerdon [b], P. Bryson [b]

[a] School of Biomedical and Biological Sciences, University of Plymouth, Drake Circus, Plymouth PL4 8AA, UK
[b] Diving Diseases Research Centre, The Hyperbaric Medical Centre, Tamar Science Park, Plymouth PL6 8BU, UK

ARTICLE INFO

Article history:
Received 19 August 2010
Received in revised form 11 February 2011
Accepted 23 February 2011
Available online xxxx

Keywords:
DNA damage
Comet assay
Human umbilical vein endothelial cells (HUVECs)
Hyperbaric oxygen
Glutathione
Hydrogen peroxide

ABSTRACT

Background: *In vitro* studies on hyperbaric oxygen (HBO) therapy suggest that HBO may cause DNA damage, but this has not been evaluated using endothelial cells.
Methods: Human umbilical cord endothelial cells (HUVECs) were exposed either to H_2O_2 or to HBO for 90 min, with or without subsequent H_2O_2 exposure. Measurements included the comet assay for DNA damage, and reduced and oxidised glutathione levels.
Results: HUVECs showed sensitivity to H_2O_2 (EC_{50} of 0.2 mM for DNA migration). A single 90 min HBO treatment at 2.2 ATA caused a statistically significant (ANOVA, $P<0.05$) increase of DNA migration in HUVECs to $6.8\pm0.3\%$ (mean ± SEM, $n=8$), which returned to normal levels ($4.9\pm0.1\%$, $n=6$) after 24 h. Further exposure to 0.2 mM H_2O_2 after HBO treatment significantly increased the DNA migration in HBO-treated cells immediately post-treatment; but 24 h later the cells showed 22% less DNA damage and higher glutathione than controls.
Conclusion: A single HBO exposure causes limited DNA damage to HUVECs, which repairs quickly. HBO treatment protects against H_2O_2-induced DNA damage and involves cellular glutathione.
Significance: Endothelial cells are unlikely to be compromised during HBO therapy.

1. Introduction

Hyperbaric oxygen (HBO) therapy involves breathing 100% oxygen at greater than 1 atmosphere absolute pressure (ATA; i.e., greater than atmospheric pressure at sea level), and has been used successfully to treat patients for wound healing (reviews, [1–3]). The mechanism of wound healing is not fully understood, but involves angiogenesis into the wound area [4]. We have recently shown that HBO does not damage the aortic endothelium of rodents *in vitro* [5]. However, some *in vitro* studies have raised concerns that HBO may cause oxidative DNA damage. Using the comet assay, a single HBO treatment has been shown to induce DNA damage *in vitro* in whole blood, human lymphocytes, A549 lung cells, and V79 Chinese hamster cells [6–9].

In vivo studies have shown more variable effects. Although patients and healthy volunteers generally showed no overt HBO-induced oxidative stress in terms of haematology and blood chemistry [10–14], induction of DNA strand breaks cannot be excluded. Some studies have shown that a single HBO exposure (2.5 ATA, 3×20 min) induces DNA strand breaks in leukocytes and lymphocytes from healthy volunteers, and these breaks may arise from oxidative damage of the bases in DNA [10,11,15]. However, the effect is transient and HBO-induced DNA damage can be apparently repaired in 2 h [15]. Several studies report no DNA damage 24 h after a first HBO treatment, or following subsequent HBO treatments [7,10,11,15]. However, the situation may be different in patients with illness (i.e., not healthy volunteers). For example, Eken et al. [16] reported persistent DNA damage to lymphocytes from patients with hypoxia-related problems even after the 10th and 20th HBO treatment (2.5 ATA, for 3×20 min in each treatment). Furthermore, most of the previous studies on humans have focused on blood cells, but effects of HBO on DNA in the endothelial cells that line blood vessels have yet to be established.

Endothelial cells are central to the success of HBO-dependent angiogenesis [17], and while there are some data suggesting DNA damage in lymphocytes, there are no data on the genotoxicity of HBO to endothelial cells. Even basic experimental information such as the responsiveness of human umbilical vein endothelial cells (HUVECs) to the common oxidising agents used as positive controls in DNA damage experiments (e.g., H_2O_2, [18]) appears to be lacking. In this study, we aim to test the effects of a single HBO treatment on HUVECs. The experimental approach included establishing positive controls with H_2O_2 to bench mark the sensitivity of HUVECs to DNA damage, as measured by the comet assay. Then a second series of experiments tested the effect of a single HBO treatment on DNA damage and

* Corresponding author. Tel.: +44 1752 584600; fax: +44 1752 584605.
E-mail address: rhandy@plymouth.ac.uk (R.D. Handy).

0304-4165/$ – see front matter
doi:10.1016/j.bbagen.2011.02.007

background level of % of DNA in comet tails (% tail DNA) was 4.2 ± 0.2% ($n = 5$), and the maximum achieved at the highest H_2O_2 concentration was 78.5 ± 3.1% ($n = 4$). The EC_{50} was 0.19 mM, and so a concentration of 0.2 mM H_2O_2 was chosen for subsequent experiments involving HBO.

3.2. Effect of HBO treatment with or without subsequent H_2O_2 exposure on levels of DNA damage

In these experiments HUVECs were treated with a single 90 min HBO treatment at 2.2 ATA, with or without a subsequent exposure to

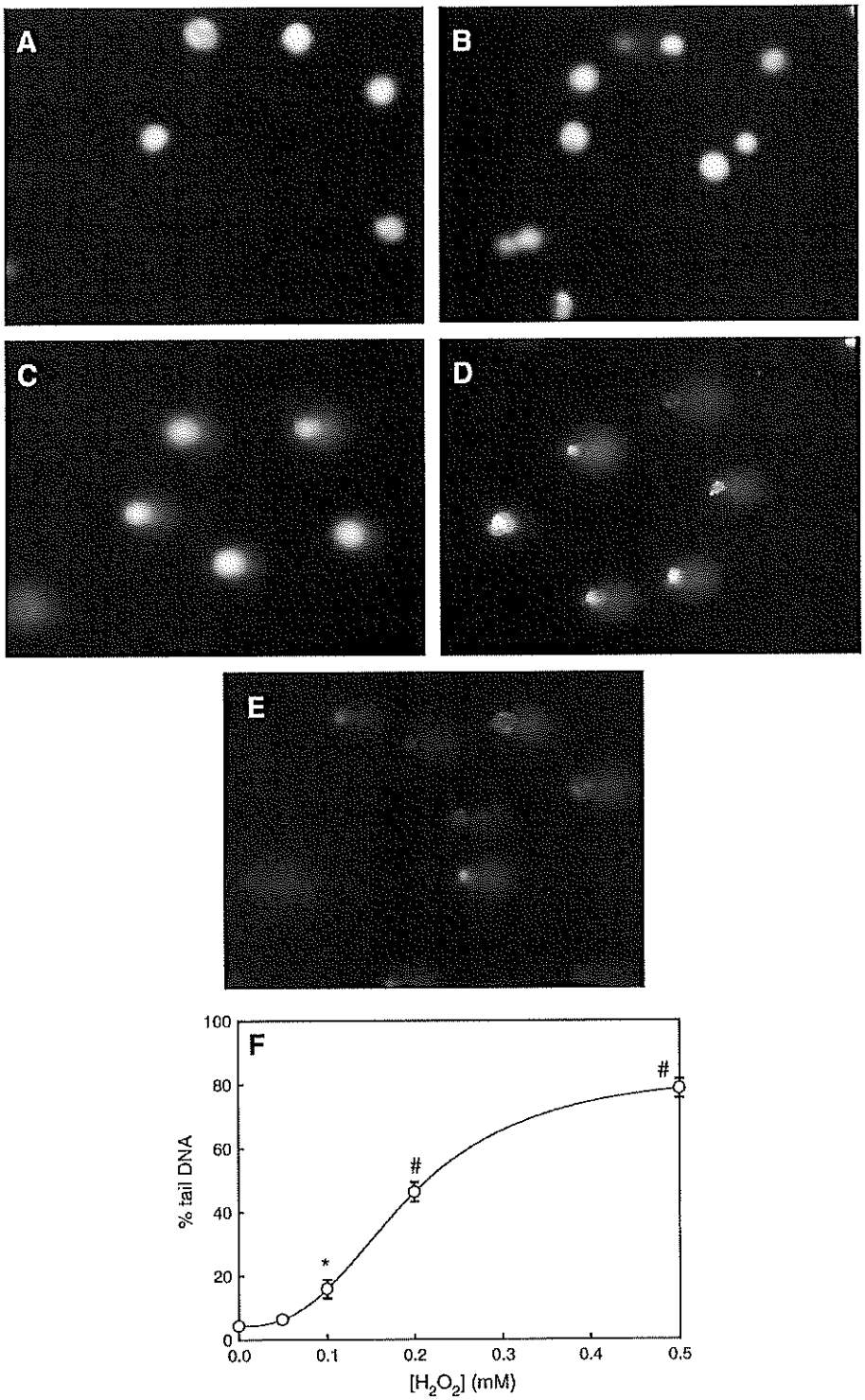

Fig. 1. Example comet images (×400 magnification) of HUVECs exposed to (A) 0 mM, (B) 0.05 mM, (C) 0.1 mM, (D) 0.2 mM, or (E) 0.5 mM H_2O_2 for 15 min at 37 °C, and the concentration response curve for % DNA tails (F). Data are means ± SEM, $n = 4$–5 replicates. * ($P < 0.05$) and # ($P < 0.001$) indicate a statistically significantly difference from the no-added H_2O_2 control (one-way ANOVA). The curve was fitted using Sigma Plot 11 (Systat Software, Inc.) using the sigmoidal function, $y = 4.3\% + 80.1\%/1 + (x/0.19)^{-2.7}$. The estimated EC_{50} from this fit is 0.19 mM.

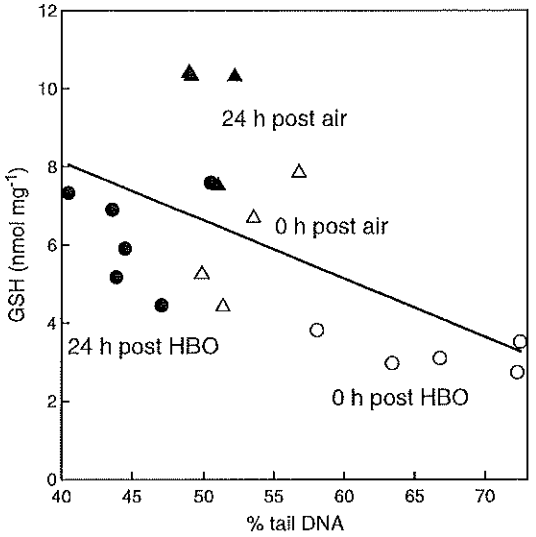

Fig. 4. The relationship between GSH levels in HUVECs after 90 min HBO or air treatment with subsequent exposure to 0.2 mM H_2O_2 for 15 min. The different data sets are shown with different symbols as indicated. The straight line is a linear regression ($r^2 = 0.311$, $P = 0.0131$).

increase of DNA in the tail of the HUVECs (Fig. 2), and the cells recovered within 24 h. In contrast to our endothelial cell cultures, *in vitro* studies using isolated blood cells that are often held in simple physiological salines can show significant DNA damage following HBO (review, [6]). For example, Dennog et al. [10] reported a 5-fold increase in DNA migration from human lymphocytes after a HBO exposure at 2.5 ATA for 3 × 20 min. Rothfuss et al. [9] also found a 6-fold increase of DNA migration in V79 cells after 1 h of HBO exposure at 3.0 ATA. It would appear that HUVECs grown in culture conditions are less sensitive to HBO-induced DNA damage than isolated blood cells.

The health of the endothelium is vital for angiogenesis during wound healing, and our *in vitro* data on cultured HUVECs supports our previous histological observations on intact blood vessels that the endothelium is not damaged by HBO therapy [5]. Our findings are also consistent with *in vivo* studies where endothelial repair and angiogenesis is well documented in patients (e.g., [3]); and studies where blood taken from volunteers 6 h or 24 h after HBO showed no effect of HBO treatment on DNA migration [15]. Overall, these data suggest the risk of DNA damage from a single HBO treatment alone, is low, and probably not of clinical importance to patient care. Further work is needed to determine if this also true for realistic multiple treatment scenarios where patients can have around 20 treatments over 2–3 weeks [12]. Dennog et al. [10] and Speit et al. [15] did find higher rates of DNA migration level in human leukocytes from healthy volunteers after multiple HBO exposure (2.5 ATA for 3 × 20 min), but the cells were quickly repaired after 1 or 2 h.

4.2. Protective effect of HBO on H_2O_2-induced DNA damage

This study shows that HBO treatment can protect HUVECs from the subsequent effects of H_2O_2, and this new finding has some important implications for patient care. Patients can have other medical treatments after HBO (e.g., drugs, surgery, etc.) and these procedures can lead to oxidative stress (e.g. [25]). In our experiment, we used H_2O_2 as a well known reference toxicant in the comet assay [23], where the hydroxyl radicals generated usually react with either the base or sugar residues of DNA to cause oxidative damage [18]. DNA damage to HUVECs had not been previously reported, and our first task was to demonstrate that HUVECs were susceptible to DNA damage, and that this reference toxicant would produce the expected concentration-dependent response. This was the case with an EC_{50} of 0.19 mM (Fig. 1). However, HUVECs do appear to be more resistant to the effects of H_2O_2 than other cell types. Collin et al. [26] found that Hela (human transformed epithelial) and GM1899A (human lymphoblastoid) cells incubated with 50 μM H_2O_2 showed considerable DNA breakage with virtually all or most comets in class 4 (equal to about 80% DNA in the tail). In contrast, freshly isolated human lymphocytes showed no damage (class 0, equal to 5% DNA in the tail approximately) at the same, and much higher (200 μM), H_2O_2 concentrations [26]. In another study using freshly isolated human lymphocytes, the EC_{50} for unwinding 50% of the DNA with H_2O_2 was about 20 μM [27]. This suggests that the freshness of the cells will influence the outcome, and arguably cells that have rested in optimal cell culture conditions (such as our HUVECs) are more likely to represent *in vivo* conditions than cells that are still suffering from the cell collection procedure.

In our experimental conditions, subsequent incubation with H_2O_2 in HBO-treated cells showed more DNA migration than that of Air-treated cells immediately post-treatment (Fig. 2). This suggests that cells can have some small and immediate vulnerability to DNA damage, but this effect is quickly outweighed by the latent beneficial effect of HBO treatment where cells showed 22% less DNA migration than that of air-treated controls by 24 h recovery (Fig. 2). Human lymphocytes *in vitro* also show similar protective HBO effects against 10–20 μM H_2O_2 at both 4 and 24 h post-HBO treatment [9]. Taken together, these observations suggest that the beneficial effects on DNA repair after HBO treatment are more important than the small theoretical risk of DNA damage during therapy. The mechanism of this latent effect is not completely clear, but probably involves changes in the cellular glutathione pool.

4.3. Effects of HBO and subsequent H_2O_2 exposure on glutathione

Reduced glutathione (GSH) plays an important role in antioxidant defences [28]. Under oxidative stress conditions, GSH is converted (dimerized) to the oxidised form of glutathione (GSSG). The GSH and GSSG content of HUVECs were about 10 nmol mg^{-1} protein and 1 nmol mg^{-1} protein, respectively (within the range of previous reports: 5.88–12.1 nmol mg^{-1} protein of GSH [29]; 10–25 nmol mg^{-1} protein of GSH and 0.2–1.2 nmol mg^{-1} protein of GSSG [30]). HBO treatment caused the utilization of intracellular GSH in HUVECs, and decreased GSSH (Fig. 3A). The former could be explained by oxidation of GSH, and the latter by the loss of glutathione from the cell (e.g., via glutathione transferase activity, [31]). Crucially, a few nmol mg^{-1} protein of both GSH and GSSH remained (Fig. 3) and therefore the antioxidant defences in the cell were not totally depleted. However, in the long term levels of GSH and GSSH need to be restored [32] and in our study the HBO-treated cells were able to recover the original glutathione pool by 24 h post-treatment (Fig. 3A).

Another important finding in our study is the protective effects of HBO treatment on H_2O_2-induced DNA damage in HUVECs (Fig. 2). The corresponding GSH measurements (Fig. 3B) were generally lower than in cells that had not been treated with H_2O_2 (Fig. 3), suggesting that some GSH is utilized in protecting the cells from H_2O_2 exposure. This is reflected in a statistically significant inverse correlation of GSH with the level of DNA migration (Fig. 4). This suggests that the utilization of the glutathione pool is instrumental in preventing the DNA damage *in vitro* in HUVECs following HBO treatment. This notion is also supported by *in vivo* observations where the GSH levels are generally preserved in blood cells from patients undergoing HBO therapy [13], and in rodents [33].

In conclusion, using the alkaline comet assay, we have demonstrated that HUVECs in culture are responsive to the reference toxicant, H_2O_2, and that concentration-dependent DNA damage occurs. However, a single 90 min HBO treatment at 2.2 ATA causes only a small, but detectable increase in DNA migration, which