Effects of hyperbaric oxygen on expression of fibrinolytic factors of human endothelium in a simulated ischaemia/reperfusion situation

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Background: Treatment with hyperbaric oxygen (HBO_2) is controversial when treating disorders other than decompression sickness. Still, HBO_2 is a treatment modality that has gained recognition in certain situations of ischaemia reperfusion. However, not much is known about its effect on the endothelial cells. Based on earlier studies, the hypothesis was that HBO_2 treatment stimulates the release of fibrinolytic factors. The aim of the study was to investigate the effect of HBO_2 treatment on cultured endothelial cells in a simulated ischaemia-reperfusion model. *Methods*: To mimic the clinical situation during ischaemia reperfusion, endothelial cells were subjected to anoxia for 8 h, followed by reperfusion with either HBO_2 or normobaric air for 1.5 h, and compared with an untreated control that was not exposed to anoxia. Components investigated were the fibrinolytic stimulator tissue plasminogen activator (t-PA), urokinase plasminogen activator (uPA) and the antagonist, plasminogen activator inhibitor type one (PAI-1). *Results*: Immediately after 8 h of total anoxia and reoxygenation with HBO_2 (for 1.5 h), the mean (SEM) concentrations of t-PA, PAI-1 and uPA were significantly increased compared to the other groups. The difference between the normobaric and control groups, measured at 1.5 h, 6 h and 24 h post-anoxia, persisted throughout the experiment. *Conclusion*: In this ischaemia-reperfusion model, HBO_2 stimulates the release of fibrinolytic factors. These observations might be relevant in trauma care in preventing thromboses and/or microembolization following ischaemia-reperfusion.

Key words: Endothelial cells; hyperbaric oxygen; PAI-1; reoxygenation; t-PA

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INTRODUCTION

The vascular endothelium is a complex modulator of a variety of biological systems. It provides an inner lining of the blood vessels and it is a dynamic participant in cellular and organ function. Endothelial cells mediate the local inflammatory response through modulation of vascular tone and local blood flow, changes in vascular permeability, induction of a prothrombotic surface and stimulation of extravasation [1]. To achieve this, endothelial cells produce,

among other substances, tissue plasminogen activator (t-PA) and plasminogen activator inhibitor type one (PAI-1), which have essential and antagonizing functions in the degradation of fibrin clots [2]. The fibrinolytical system plays an important role in the patency of the vasculature, and PAI-1 and t-PA are regulators of fibrinolytic activity [3]. Elevated levels of PAI-1 have been associated with thrombotic and prothrombotic states [4].

Trauma often compromises the arterial circulation directly by damaging the vessel, or indirectly through chock-associated hypoperfusion, which together with the formation of thrombi might eventually lead to ischaemia and/or total anoxia of the tissues involved. If there is a delay between the trauma and the reperfusion, the risk of ischaemia-reperfusion syndrome increases, and the affected tissue might succumb to necrosis.

Hyperbaric oxygen treatment (HBO_2) is a well-established method of treating a variety of clinical disorders related to trauma, e.g. crush injuries, complicated wounds [5] and gas gangrene [6]. Contemporary investigations have focused on the effect of hyperbaric oxygen on leukocytes [7–9]. However, oxygen has been demonstrated to have a multitude of functions, including regulation of endothelial cell fibrinolytic capacity [10]. This might be of importance in keeping and re-establishing vascular patency. In this study, the hypothesis was that HBO_2 stimulates the release of fibrinolytic factors. The hypothesis was tested in an ischaemia-reperfusion model on cultured human endothelial cells.

MATERIALS AND METHODS

Endothelial cells

Endothelial cells from human adult saphenous veins (HAVEC) were isolated by a modified collagenase (0.1%, Sigma, St. Louis, MO, USA) digestion according to Jaffe *et al.* [11]. Cells were cultured in E199 medium supplemented with 1.1 mmol/L L-glutamine, 32 klU/mg/L penicillin-streptomycin, 20% fetal calf serum, 150 mg/L endothelial growth factor and 10 kIU/L heparin on 0.2% gelatine coated culture plates. The cells were cultured at + 37°C in 5% CO_2. The endothelial origin of the cells was verified by their typical cobblestone morphology in phase contrast microscopy, and by positive immunofluoroscence staining with an anti-human von Willebrand factor antibody (A 082, DAKO A/S, Copenhagen, Denmark). To obtain a sufficient amount of cells, the cultures were passaged three times. The HAVECs were cultured in three 24-well plates, and a total of 6 wells were used in each of the 3 experimental groups (n = 6) at each time point. HAVECs were taken from the incubator immediately prior to the experiment and the medium was changed prior to the experiment.

Experimental design

To investigate the effects of HBO_2 in an ischaemia and reperfusion model on endothelial cell function, the following design was used. Group I: total anoxia for 8 h followed by 1.5 h of HBO_2; Group II: total anoxia for 8 h followed by normobaric air for 1.5 h; Group III: this group served as an untreated control, exposed neither to anoxia nor to HBO_2. Anoxia and HBO_2 were administered in a small compression chamber manufactured by the Swedish National Defence Research Establishment. HBO_2 was induced at 250 kPa with 100% oxygen (O_2). Anoxia was induced by 100% nitrogen (N_2) at atmospheric pressure (100 kPa). The controls were kept during similar ambient conditions but outside the chamber. Immediately after exposure to anoxia (at 0 h), post-exposure at 1.5 h, 6 h and 24 h, media were withdrawn and frozen at – 70°C in aliquots. An identical sampling schedule was used for the untreated controls. Total mRNA from the cell was isolated at 1.5 h and 24 h. The rationale for administering HBO_2 for 1.5 h is that it is a common treatment regimen at many hyperbaric facilities. The study was approved by the local ethics committee.

Protein secretion

The concentration of fibrinolytic components in conditioned media was assayed using enzyme-linked immunosorbent assays (ELISA) available commercially from Biopool AB, Umeå, Sweden. The following kits were used: PAI-1 antigen was analysed using Imulyse PAI-1, t-PA antigen was analysed using Imulyse t-PA, and uPA antigen was analysed using uPA ag EUMIX-5

(Monozyme, Hoersholm, Denmark). The fraction of active t-PA was assayed using Funktionell t-PA (Novo Nordisk, Baegsvaerd, Denmark). The assay characteristics have been described earlier [12, 13].

Gene expression

Total mRNA was isolated from cultures of the different treatment groups and used to determine the expression of t-PA, PAI-1 and uPA transcripts. The isolation was carried out using the guanidine/isothiocyanate/phenol/chloroform method (Trizol, Life Technologies, Grand Island, New York, USA). To obtain enough mRNA for the RT-PCR analyses, the mRNA was pooled from the six cell cultures in the same treatment group. Gene transcripts for t-PA, PAI, uPA and glycenaldehyde-3-phosphate dehydrogenase (G3PDH) were determined as previously described [13, 14].

Statistics

The differences in specific protein concentration in conditioned media between the three groups were analysed using the unpaired, non-parametric Kruskal-Wallis test. If there was an overall difference, comparisons between groups were done using the Mann-Whitney U-test. P-values less than 0.05 were considered significant. Values are expressed as mean (SEM).

RESULTS

Fibrinolytical factors

Following anoxia. Immediately after 8 h of anoxia, there was a significant difference in t-PA concentration among the three groups ($p < 0.05$) in that t-PA concentration was higher in the cultures that were subjected to anoxia. There was no significant difference between the anoxic groups. Also, PAI-1 differed among the experimental groups after the anoxic period, being significantly ($p < 0.05$) higher in media from cells that had been anoxic (Groups I and II), with no significant differences between these two cultures compared to the control (Group III). Similarly, uPA concentration increased in media from cultures that had been deprived of O_2 for 8 h, being 2–3 times higher in Group I and Group II compared with the control (Group III).

Following HBO_2 treatment or exposure to normobaric air—t-PA. Immediately after HBO_2 treatment (Group I) the mean concentration of t-PA in conditioned media was significantly higher than in the other groups ($p < 0.05$), being 4 times higher than in Group III and 2.5 times higher than post-anoxic cultures that were exposed to normobaric air alone Group II (Fig. 1). At this time point, there was no longer a statistically significant difference between the normobaric group (Group II) and the untreated control (Group III). The cells expressed t-PA transcripts that were most abundant in Group I, followed by Group II and Group III (Fig. 4).

The difference persisted ($p < 0.05$) 6 h after anoxia (4.5 h after HBO_2 treatment). Mean concentration of t-PA was three times greater in the HBO_2-treated group compared with the cultures that were exposed to normobaric air. There was no significant difference between Groups II and III (Fig. 1).

A similar difference persisted up to 24 h after anoxia (22.5 h after HBO_2 treatment) among the three groups with 3–4 times greater t-PA concentration in media ($p < 0.05$) from HBO_2-treated cells (Group I), compared with Group II, or untreated controls (Fig. 1). At this time point, the cell cultures no longer expressed t-PA transcripts above the detection limit (Fig. 4).

PAI-1. One-and-a-half-hours post-anoxia there was still a significant difference in PAI-1 concentration in cell culture media ($p < 0.05$). HBO_2

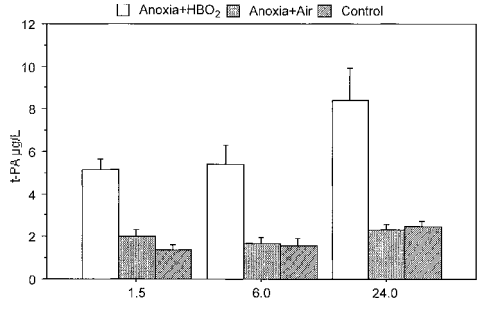

FIG. 1. Mean (SEM) concentration of t-PA in conditioned media of endothelial cells subjected to an anoxic state of 8 h followed by reoxygenation with HBO_2 (Anoxia + HBO_2) normobaric air (Anoxia + Air) compared with a control. HBO_2 treatment resulted in an immediate and transient increase in t-PA in conditioned media significantly higher than in the other two groups. There were no significant differences between the normobaric and control groups.

treatment (Group I) resulted in a 3-fold increase in mean PAI-1 concentration in conditioned media from cells that had been exposed to anoxia. There were no significant differences though between Group II and Group III at this time (Fig. 2). PAI-1 transcripts were a 100-fold lower in the cultures that had been subjected to anoxia alone (Group II), and a 1000-fold lower in cultures that were subsequently exposed to HBO_2 compared to Group III.

Six hours post-anoxia, the PAI-1 was differentially expressed in media from the three groups ($p < 0.05$). The highest concentration was found in the HBO_2-treated cells in Group I, being eight times greater than in cells that, post-anoxia, were exposed to normobaric air, Group II, and five times higher than in the untreated controls in Group III. The untreated controls in Group III had at this time accumulated more PAI-1 in their media than Group II ($p < 0.05$, Fig. 2).

After 24 h the difference was attenuated, but persisted, PAI-1 concentration was three times higher in the HBO_2-treated cultures in Group I, compared with cells that had been exposed to anoxia followed by normobaric air, Group II, and two times higher than in the untreated controls, Group III ($p < 0.05$). Similarly, the PAI-1 concentration was still higher in Group III than in Group II ($p < 0.05$, Fig. 2). At this time there were no detectable PAI-1 gene expression (Fig. 4).

UPA. One-and-a-half-hours post-anoxia there was a significant difference in uPA concentration in cell culture media from the three groups ($p < 0.05$). The highest concentration was observed in the HBO_2-treated Group I, being three times that of Group II, and twice as high as Group III. There was no statistically significant difference between the normobaric group (Group II) and the controls (Group III) (Fig. 3). Gene transcripts of uPA were most abundant in Groups II and III and 10-fold lower in cultures that had been exposed to HBO_2 (Group I) (Fig. 4).

The difference persisted 6 h after anoxia ($p < 0.05$), with the highest uPA concentration observed in the HBO_2-treated cells in Group I and almost twice as high as in Group II and Group III. Here were no differences between Groups II and III (Fig. 3).

The difference between the three groups persisted 24 h after anoxia ($p < 0.05$), again being highest in the HBO_2-treated cells in Group I. These cultures expressed 2–3 times as much uPA as Group II or Group III. At this time the untreated controls in Group III had released more uPA into the media than cells that had been exposed to anoxia followed by normobaric air, Group II ($p < 0.05$, Fig. 3). After 24 h the level of uPA transcripts had not changed in the HBO_2-treated Group I, whereas it was lowered (Group III), or absent (Group II) in the other groups (Fig. 4).

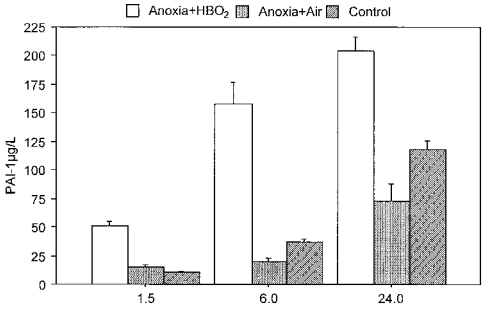

FIG. 2. Mean (SEM) concentration of PAI-1 in conditioned media of endothelial cells subjected to an anoxic state of 8 h followed by reoxygenation with HBO_2 (Anoxia + HBO_2) or normobaric air (Anoxia + Air) compared with a control. HBO_2 treatment resulted in an immediate and transient increase in PAI-1 in conditioned media significantly higher than in the other two groups. After 6 h the control group had accumulated significantly more PAI-1 than the normobaric group, and this difference persisted after 24 h.

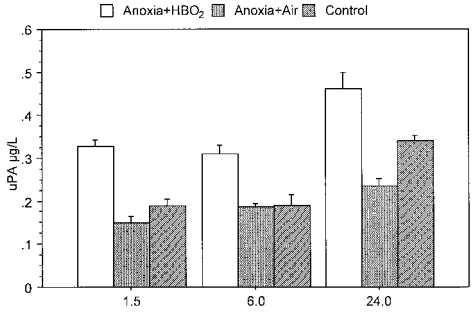

FIG. 3. Mean (SEM) concentration of uPA in conditioned media of endothelial cells subjected to an anoxic state of 8 h followed by reoxygenation with HBO_2 (Anoxia + HBO_2) or normobaric air (Anoxia + Air) compared with a control. HBO_2 treatment resulted in an immediate and transient increase in uPA in conditioned media significantly higher than in the other two groups. After 24 h the concentration of uPA in the control group was significantly higher than in the normobaric group.

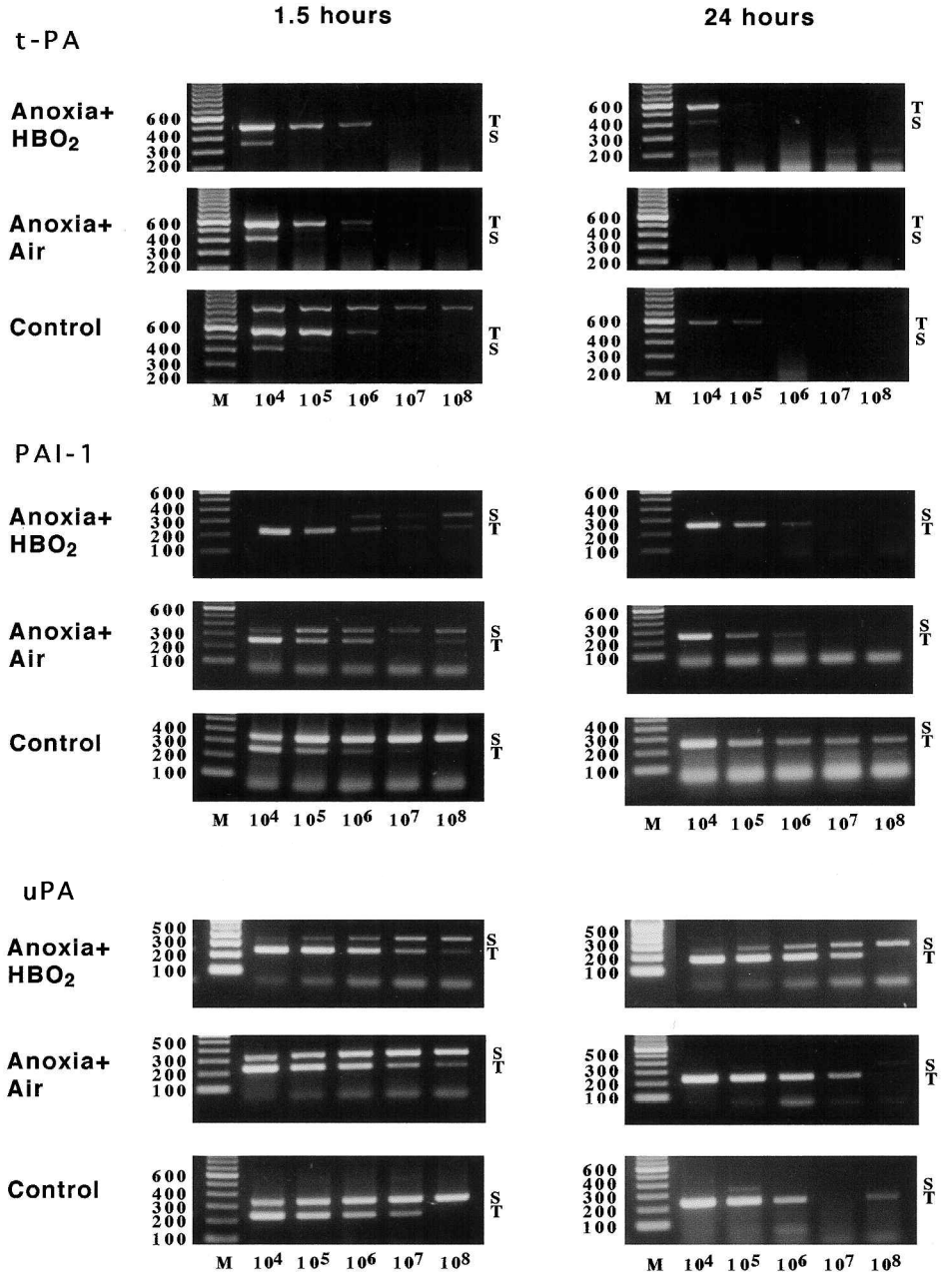

FIG. 4. Agarose gel displaying competitive quantitative RT-PCR analysis of t-PA, PAI-1 and uPA at 1.5 and 24 h after anoxia, following treatment with HBO_2, normobaric air or untreated control. The different products were separated on 1.8% agarose gels, stained with ethidium bromide and photographed. The upper bands are the PCR products generated from the specific message in total cellular RNA. The lower bands are from the external cRNA (shown from left to right at dilutions corresponding from 10^4 to 10^8 copies per microgram of total RNA). The multiple lanes at the far left are the 100 base pair DNA markers (M = Molecular Marker, T = Template, S = Sample).

DISCUSSION

In the present study, anoxia appears to stimulate the endothelial cells to produce and secrete t-PA, PAI-1 and uPA, compared with endothelial cells that have not been provoked by anoxia. This effect was then augmented after reoxygenation with HBO_2. This effect was not seen after reoxygenation with normobaric air, where the fibrinolytic response resulted in low concentrations of t-PA, PAI-1 and uPA.

Specific mRNA for t-PA, PAI-1 and uPA was detectable in all three groups at 1.5 h, indicating an ongoing transcription in all experimental groups. The expression of t-PA, however, was most pronounced in the HBO_2-treated group and PAI-1 transcripts were most abundant in the normobaric group. After 24 h, specific mRNAs were only detectable for uPA in the HBO_2 and control groups, suggesting that these factors were differentially regulated by anoxia and HBO_2. In the HBO_2-treated group, which had the largest concentrations of the fibrinolytic proteins, the net amount of t-PA was at all times higher than the other proteins. At the transcription level, HBO_2 treatment constituted a strong stimulus to produce t-PA, whereas in the normobaric group the effect was most pronounced for PAI-1. In fact, HBO_2 treatment resulted in high secretion of all the fibrinolytic factors but evidently constituted a low stimulus to produce PAI-1. However, *in vivo*, t-PA binds to the fibrin mesh during thrombolysis, which renders it inaccessible to inactivation by PAI-1 and increases its activity 1000-fold [15]. Clinically, this might lead to HBO_2 treatment exerting a protective function regarding the development and persistence of microemboli.

Accumulation of t-PA, PAI-1 and uPA in the conditioned medium represents the sum of the constitutive protein synthesis and the acute-release from cellular stores. The cell, or matrix-bound pools, of t-PA has been demonstrated to be fairly small, and the acute-release from cellular stores to be strongly correlated to the protein synthesis [16]. The fact that the normooxic group did not show any elevation in the concentrations of proteins following anoxia and reoxygenation, and even statistically significantly lower amounts of PAI-1 and uPA at certain time points than the control group, could be interpreted as a relative decline in the synthesis or release. The present study demonstrates that when endothelial cells are reoxygenated with hyperbaric oxygen there is no decrease in the secretion of proteins at any time point investigated. On the contrary, there was a sustained increase in the secretion of all the proteins following the hyperoxic reoxygenation throughout the experiment. This could indicate that an elevated oxygen partial pressure exerts a beneficial effect on the endothelial cells after ischaemia-reperfusion, an effect similar to that of superoxide dismutase [17].

Hyperoxia is known to be toxic to the lungs and to the CNS, but it is also feared that it exacerbates ischaemia-reperfusion injuries by adding extra oxygen to the system and thus increasing free radical production. If this hypothesis is true, it would be harmful to treat patients under these circumstances with higher partial pressures of oxygen. This notion is supported by ischaemia-reperfusion studies on rats treated with HBO_2, studies demonstrating that if the oxygen partial pressure was higher than the atmospheric pressure then the positive oxygen effects exceed the expected negative oxygen effects [7, 9, 18]. The exact mechanisms for this are not known, although some studies have shown that HBO_2 inhibits leukocyte β_2-integrin function and thus cell adherence [8], which might be important in the mediation of ischaemia-reperfusion injury. Nevertheless, combined with the present findings, these observations strongly suggest that HBO_2 might be beneficial in the post-anoxic period.

We have previously reported that HBO_2 treatment stimulates cultured endothelial cells to produce and secrete elevated levels of t-PA and PAI-1 compared to hyperbaric air, indicating that increased oxygen partial pressure, and not increased pressure, is an important component [10]. In this study we focused on the effect of HBO_2 treatment after anoxia. Although the situation *in vivo* exhibits higher complexity, with constant interactions between endothelial cells and circulating blood, our results suggest that hyperbaric oxygen exerts certain effects on the fibrinolytic response after anoxia/ischaemia that might be beneficial. To clarify if this has any clinical relevance, however, will need further investigation

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