Eur. J. Clin. Chem. Clin. Biochem.
Vol. 32, 1994, pp. 279–284

In Vitro Effects of Hypoxia and Reoxygenation on Human Umbilical Endothelial Cells[1]

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(Received May 17/August 6, 1993)

Dedicated to Prof. Dr. Dr. Helmut Greiling on the occasion of his 65th birthday

Summary: We investigated metabolic changes in human umbilical venous endothelial cells, when these were incubated under hypoxic followed by hyperoxic conditions, thus simulating hypoxia and reoxygenation. The human umbilical venous endothelial cells were incubated with a degassed buffer (oxygen content: 0–0.5%) for either 3 h or 24 h, followed by a 60 min incubation with oxygen-perfused buffer (oxygen content: 100%). Three hours of hypoxia led to a slight decrease in the ATP and creatine phosphate content (−16% ± 5%), while a pronounced decrease of high energy phosphates (−54% ± 4%) was observed after 24 h of hypoxia. Reoxygenating the cells after 3 h of hypoxia led to restoration of the content of high energy phosphates, while reoxygenation after 24 h resulted in a strong decrease (−66% ± 4%). The prostaglandin I_2 release during the first 3 h of hypoxia exceeded the release in the following 21 h. In all cases, reoxygenation increased the prostaglandin I_2 release. Under normoxic conditions the ratio between oxidised glutathione and reduced glutathione shifted from 1 : 100 to 1 : 4.5 after 3 h of hypoxia. The content of lipid peroxidation products was almost unaffected during hypoxia, whereas reoxygenation resulted in a pronounced increase (+ 380% ± 60%). The results of this in vitro study suggest that relatively long periods of hypoxia lead to a deficiency of high energy phosphates in the cell. Reoxygenation leads to the formation of oxygen-derived radicals, irrespectively of a prior hypoxia.

Introduction

Oygen-derived radicals play an essential role in tissue damage (1). The potential sources of toxic oxygen species generated during ischaemia and reoxygenation included prostaglandin biosynthesis, mitochondrial electron transport systems, purine catabolism by means of xanthine oxidase and infiltration by phagocytes (2–6).

It is generally accepted that hypoxia and reoxygenation increase the formation of oxygen-derived free radicals. Modifications of intracellular enzymes and membranes result from the exhausted capacity of the antioxidant defence mechanisms (7). Previous in vitro investigations used radical-generating systems such as xanthine/xanthine oxidase or hydrogen peroxides (H_2O_2) to study the effects of radicals on cell metabolism (8–10). Our present study, using human umbilical venous endothelial cells investigates the effects of hypoxia on cell metabolism.

The influence of hypxia and reoxygenation on the intracellular content of high energy phosphates, the release of lactate dehydrogenase, glucose consumption, the ratio between reduced and oxidised glutathione and formation of lipid peroxidation products were determined. In addition, possible effects on prostaglandin I_2 production were evaluated, since it was reported that an initial release of fatty acids from membrane phospholipids occurs during ischaemia, due to phospholipase activation and ATP-dependent fatty acid acylation inhibition (11).

[1]) This project was supported by the "Medizinisch-Wissenschaftlicher Fonds des Bürgermeisters der Bundeshauptstadt Wien".

Material and Methods

Cell culture

Endothelial cells were prepared using human venous umbilical veins. Cells were isolated and cultured according to a modified standard procedure reported by *Jaffe* (9). Briefly, fresh human umbilical veins were filled with 1 g/l collagenase solution and incubated at 37 °C for 5 min. Thereafter the veins were perfused with M 199 (Gico, USA) medium containing 200 ml/l human serum (pH 7.4). Cells were collected from the perfusate by centrifugation at 800 *g* for 5 min and seeded into culture T-75 flasks precoated with human fibronectin (Biomedica, Austria). Cells were cultured in M 199 medium (pH 7.4) containing 200 ml/l human serum, 100 000 U/l low molecular weight heparin (Sigma, Germany) and 30 mg/l bovine hypothalamic growth factor (Biomedica, Austria). The confluent primary monolayers were washed and trypsinized. Cell suspensions were transferred either into the wells of a 24-well culture plate (determination of ATP, creatine phosphate and prostaglandin I_2) or the wells of a 6-well culture plate (determination of thiobarbituric acid reactive substances, reduced glutathione and oxidised glutathione). Cells were cultivated for four days. Only confluent cells from these first subcultures (24-well culture plate: approx. 140 000 cells/well, 6-well culture plate: approx. 580 000 cells/well) were used for the experiments described below. The cells were identified as endothelial cells by the typical cobblestone, contact-inhibited morphology (13) and by factor VIII (14) staining.

Exposure of cells to hypoxia and reoxygenation

The culture medium was removed and the cell layers were gently washed with preheated (37 °C) phosphate-buffered saline (containing 2.2 mmol/l Ca^{2+}, 0.5 mmol/l Mg^{2+} and 5 mmol/l glucose). A degassed phosphate buffered saline was used for the incubation, which had been perfused with 100% N_2 for 20 min. The O_2 content was approx. 0–0.5%. The culture plates were placed in a modified incubator which allowed handling under low oxygen tension (1–2% O_2), which was monitored by means of a WTW Oxi96 oximeter (WTW, Germany). The cell layers were incubated with the degassed and perfused buffer. After incubation the culture plates were placed in an absolutely airtight chamber filled with 100% N_2 for 3 and 24 h, respectively. The oxygen tension in the medium closely paralleled that in the gas phase. All experiments were performed at 37 °C. At the end of the incubation the plates were divided into two groups. One group (hypoxia group) was used to determine the metabolic effects of hypoxia (3 and 24 h). For the second group (hypoxia reoxygenation group) phosphate buffered saline was perfused with 100% O_2 for 20 min, then used to simulate a 60 min reoxygenation after hypoxia. The O_2 content of the buffer was four times higher than normal (35 versus 8.8 mg/l O_2). Cell culture plates were incubated and placed in the chamber for 60 min. The chamber was filled with 100% O_2. Control experiments for both groups were incubated with air-equilibrated phosphate buffered saline (normoxia group) for the same incubation period. To investigate the effects of a high oxygen content, human umbilical venous endothelial cells were also incubated with the hyperoxic phosphate buffered saline (normoxia/reoxygenation group). The pH was recorded after every hypoxic and reoxygenation exposure and remained stable.

Determination of intracellular ATP and creatine phosphate content

Intracellular ATP and creatine phosphate contents were determined using cell lysates obtained by the addition of 250 µl of 0.5 mol/l $HClO_4$ to each well. Lysates were neutralised with a 1 mol/l K_2CO_3 solution, transferred into cups, centrifuged and supernatants analysed for ATP and creatine phosphate. Creatine phosphate was determined after conversion to ATP using creatine kinase, with ADP as substrate and N-acetylcysteine as an activator. ATP was measured by bioluminescence using an ATP-monitoring reagent and a 1251-Luminometer (LKB, Sweden) as described earlier (15).

Determination of glucose consumption and lactate dehydrogenase

Glucose and the activity of lactate dehydrogenase in the incubation buffer were measured photometrically on an Ektachem XR700 (Kodak, USA) analyser.

Determination of prostaglandin I_2 release

At the end of the incubations the supernatants of the cell layers were transferred into micro-test tubes containing indomethacin (30 µmol/l final concentration) to prevent any further eicosanoid formation by single detached cells; the tubes were stored at −70 °C until analysis. The stable degradation product of prostaglandin I_2, 6-keto-prostaglandin $F_{1\alpha}$, was measured by a radioimmunoassay (RIA) from BIOTECX, USA. Neither the buffer itself nor any of the agents used in these experiments showed interference with the RIA. Reliability and specificity of the assays were confirmed, using the cyclooxygenase inhibitor, indomethacin. The cross-reactivities of the RIA were reported recently (16).

Determination of intracellular reduced glutathione and oxidised glutathione

The simultaneous determination of reduced glutathione and oxidised glutathione was carried out using selectively active enzymes as described in l. c. (17). Cells were lysed by adding 750 µl of 0.5 mol/l $HClO_4$ to each well. The lysates were neutralised with a 1 mol/l K_2CO_3 solution, the cell suspensions of 3 wells were pooled, transferred into cups, then centrifuged, followed by determination of reduced glutathione and oxidised glutathione. Reduced glutathione was determined photometrically as S-lactyl-glutathione after converting reduced glutathione into S-lactyl-glutathione using glyoxalase-I in the presence of methylglyoxal. Oxidised glutathione was determined photometrically by the decrease of added NADPH in the presence of glutathione reductase.

Determination of lipid peroxidation products

The phosphate buffered saline was removed from the cell layers and 1 ml 20 mg/kg aqueous butyl-hydroxy-anisol solution was added to each well. Lipid peroxidation products were determined (18) as thiobarbituric acid reactive substances.

Statistical analysis

Values are expressed as mean ± SD. For the determination of the statistical significance a matched pairs-T-test was used.

Results

ATP, creatine phosphate and glucose

Three hours of hypoxia had no effect on the ATP content. However, the creatine phosphate content decreased by 23.9% compared with controls. Subsequent hyperoxic conditions for 60 min resulted in a restoration of the creatine phosphate content, whereas ATP remained stable (tab. 1). Twenty four hours of hypoxia had a pronounced effect on the content of high energy phosphates. Both ATP and creatine phosphate concentrations decreased dramatically (tab. 2). A 60 min period of re-

Tab. 1 Contents of ATP, creatine phosphate and thiobarbituric acid reactive substances after 3 h of hypoxia and 3 h hypoxia followed by 60 min of reoxygenation.

		Normoxia group 3 h	Hypoxia group 3 h	Normoxia/ reoxygenation group 3 h + 60 min	Hypoxia/ reoxygenation group 3 h + 60 min
ATP	nmol/10^6 cells	10.8 ± 0.3	11.0 ± 0.5	11.9 ± 0.4	10.4 ± 0.5
Creatine phosphate	nmol/10^6 cells	20.5 ± 1.4	15.6 ± 2.3*	22.0 ± 4.6	23.0 ± 3.2#
Thiobarbituric acid reactive substances	pmol/10^6 cells	33 ± 2	36 ± 4	135 ± 26*#	145 ± 8*#

* = statistically significant compared to normoxia group ($p < 0.001$)
\# = statistically significant compared to hypoxia group ($p < 0.0010$
Values are given as mean ± SD (n = 12).

Tab. 2 Contents of ATP, creatine kinase and thiobarbituric acid reactive substances after 24 h of hypoxia and 24 h hypoxia followed by 60 min of reoxygenation.

		Normoxia group 3 h	Hypoxia group 3 h	Normoxia/ reoxygenation group 3 h + 60 min	Hypoxia/ reoxygenation group 3 h + 60 min
ATP	nmol/10^6 cells	10.6 ± 0.6	4.9 ± 0.3*	11.7 ± 0.5	6.2 ± 0.5*
Creatine kinase	nmol/10^6 cells	19.8 ± 5.1	9.6 ± 1.0*	21.2 ± 1.5	5.3 ± 1.5*#
Thiobarbituric acid reactive substances	pmol/10^6 cells	35 ± 4	46 ± 6	140 ± 20*#	147 ± 20*#

* = statistically significant compared with normoxia group ($p < 0.001$)
\# = statistically significant compared with hypoxia group ($p < 0.001$)
Values are given as mean ± SD (n = 12).

oxygenation following 24 h of hypoxia slightly increased the ATP content compared with the hypoxic state. In contrast, the creatine phosphate content decreased further. Thus, an overall decrease of total high energy phosphate was observed (tab. 2). No significant differences in the ATP and creatine phosphate contents were detectable after 60 min of reoxygenation without hypoxic pretreatment, in comparison with control experiments. Basic glucose consumption during 3 h of normoxic and hypoxic conditions were 1.5 ± 0.4 and 1.5 ± 0.5 µmol/10^6 cells, respectively. Twenty four hours of incubation increased the glucose consumption of normoxic cells and had an even greater effect on hypoxic cells: 2.2 ± 0.6 and 4.5 ± 0.9 µmol/10^6 cells, respectively.

Release of lactate dehydrogenase

Three and 24 h of hypoxia resulted in slight increase of the extracellular lactate dehydrogenase activity, although these increases were not statistically significant (fig. 1). In contrast, reoxygenation led to a strong increase in the lactate dehydrogenase release. Similar effects were observed with and without hypoxic pretreatment. The highest extracellular lactate dehydrogenase activity was found after 24 h hypoxia followed by reoxygenation, but this was not significantly different from the corresponding normoxic controls.

Prostaglandin I_2

Duration of hypoxia played a key role in prostaglandin I_2 release. Three hours of hypoxia led to an increase of prostaglandin I_2 levels to values approximately 240% higher than those determined in normoxic control experiments. Twenty four hours of hypoxia further enhanced the total prostaglandin I_2 release.

Reoxygenation for 60 min led to a pronounced increase in prostaglandin I_2 release. Hypoxic pretreatment diminished this "reperfusion release". The results are shown in figure 2.

Reduced glutathione and oxidised glutathione

Under normoxic conditions the ratio between oxidised glutathione and reduced glutathione was 1 : 100 (fig. 3). After 3 h of hypoxia the oxidised glutathione : reduced glutathione ratio shifted to 1 : 4.5, the subsequent reoxygenation led to a further increase in oxidised glutathione (oxidised glutathione : reduced glutathione ratio: 1 : 2.4). After 24 h of hypoxia no intracellular reduced glutathione could be detected (data not shown).

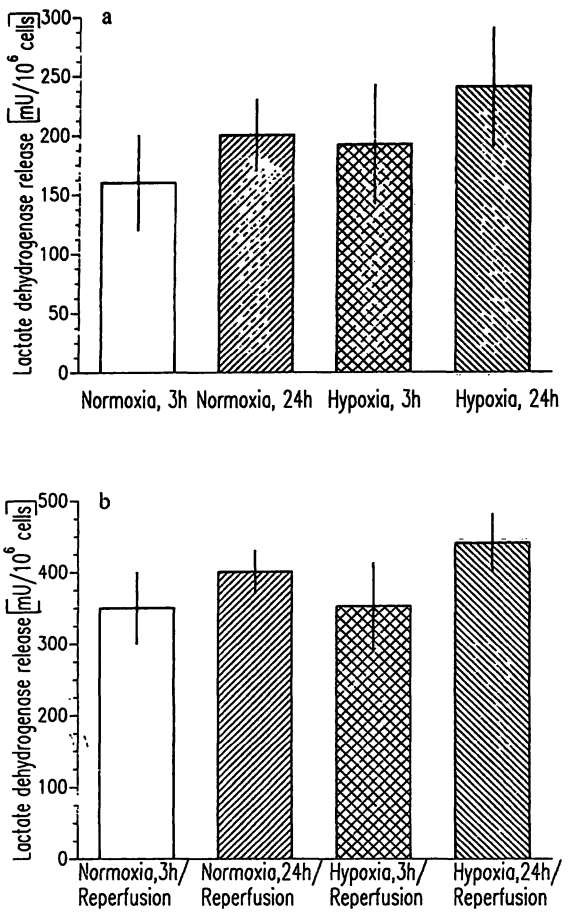

Fig. 1 Lactate dehydrogenase release from human umbilical venous endothelial cells after 3 h and 24 h of hypoxia before (a) and after 60 min of reoxygenation (b), n = 11.

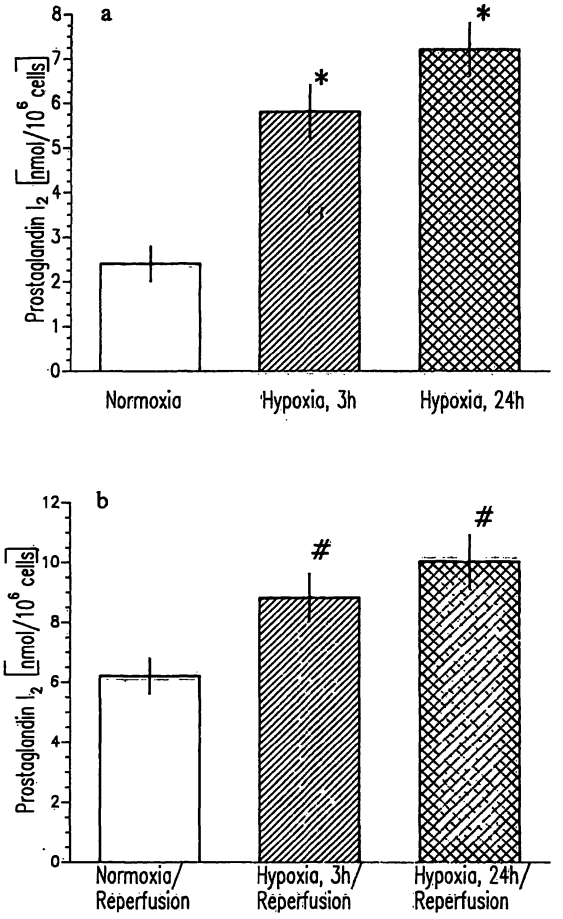

Fig. 2 Prostaglandin I_2 (measured as 6-keto-prostaglandin $F_{1\alpha}$) release from human umbilical venous endothelial cells after 3 h and 24 h of hypoxia before (a) and after 60 min of reoxygenation (b). Significant differences compared with normoxic controls: * = ($p < 0.001$), # = ($p < 0.05$), n = 10.

Thiobarbituric acid reaction substances

Three and 24 h of hypoxia resulted in slight increases of the thiobarbituric acid reactive substance levels, although no statistically significant differences from the control experiments were computable. Reoxygenation led to an enhancement of thiobarbituric acid reactive substances production. This effect was observed with and without hypoxic pretreatment (tab. 1, 2).

Discussion

The aim of this study was to determine cellular metabolic conditions after different periods of hypoxia or normoxia followed by reoxygenation. Thiobarbituric acid reactive substance formation as a measure for oxygen-derived free radical activity was also investigated. This was of special interest, since in contrast to the endothelial cells of many other species, human endothelial cells exhibit no measurable activities of xanthine oxidase using radioactive substrates under normoxic, hypoxic and hyperoxic conditions (19). Xanthine oxidase is reported to be one of the main sources for the intracellular produced oxygen derived free radicals during tissue hypoxia (6).

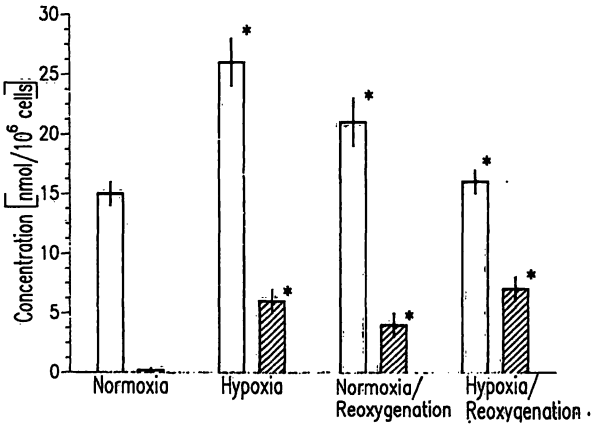

Fig. 3 Intracellular content of reduced glutathione (□) and oxidised glutathione (▨) in human umbilical venous endothelial cells after 3 h hypoxia followed by 60 min of reoxygenation.
* = Significant differences compared with normoxic controls ($p < 0.001$), n = 9.

Long periods of hypoxia (3 h, 24 h) were necessary, since in contrast to physiological conditions, cultured

endothelial cells are not exposed to shear stress, thus requiring a lower level of metabolic activity to maintain their function. The lack of oxygen leads to massive changes in the cellular steady state. This was supported by the fact that glucose consumption showed marked changes only after 24 h of hypoxia, indicating the change from oxidative phosphorylation to anaerobic glycolysis (20), and resulting in a reduced production of high energy phosphates. The creatine phosphate content reacted more sensitively than ATP, which decreased after longer periods of hypoxia. This is in accordance with the results of *Jennings* et al. from the study of myocardial ischemia (21).

Although *Madden* et al. (22) reported the inactivation of cyclooxygenase under hypoxic conditions, we observed an increased prostaglandin I_2 release during hypoxia. One explanation could be the activation of phospholipases by hypoxia, resulting in an increased degradation of phospholipids (23, 24). During the first 3 h of hypoxia more prostaglandin I_2 was released than in the following 21 h. Similar results were obtained by *Palluy* et al. (25) and also by *Farber* et al. (26), who suggest a transient activation of cyclooxygenase by hypoxia. Although thiobarbituric acid reactive substance formation was increased, reoxygenation was rapidly answered by the release of prostaglandin I_2, which seemed contrary to the well established opinion that low levels of thiobarbituric acid reactive substances stimulate and high levels inhibit the activation of cyclooxygenase (27, 28). Experiments using H_2O_2 as an oxygen radical generating system showed a release of prostaglandin I_2 concomitant with increasing rates of lipid peroxide formation (29). Therefore it seems obvious that the onset of reoxygenation activates cyclooxygenase and longer periods of reoxygenation depress cyclooxygenase activity.

As mentioned above, reoxygenation after 3 h of hypoxia induced an increased consumption of reduced glutathione, a further increase in oxidised glutathione and the restoration of total high energy phosphates. A different situation occurred when 24 h of hypoxia was followed by reperfusion. No reduced glutathione could be detected and the total high energy phosphate content was dramatically reduced. *Schimke* et al. have shown that increasing concentrations of H_2O_2 primarily lead to an increased content of high energy phosphates followed by a breakdown (30). Therefore injury during reoxygenation is probably due to enhanced radical production. If the antioxidative capacity and cellular energy pools are exhausted by longer periods of hypoxia, reoxygenation will lead to irreversible cell damage. In contrast, the hyperoxic conditions used in this study induced thiobarbituric acid reactive substance formation without prior hypoxia. This strongly suggests that the antioxidative capacity of the normoxic cells is not sufficient to prevent the general lipid peroxidation induced by exposure to high concentrations of oxygen.

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