Effects of Hypoxic Gas Mixtures on Viability, Expression of Adhesion Molecules, Migration, and Synthesis of Interleukins by Cultured Human Endothelial Cells

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The effects of short-term (3 h) and long-term (18 h) repeated hypoxic exposure (5% O_2) on viability of endothelial cells, expression of adhesion molecules, and secretion of IL-6 and vascular endothelial growth factor were studied. The resultant number of apoptotic and necrotic cells indicates that cultured endothelium well tolerates repeated changes in partial oxygen pressure in the medium, despite the stress reaction manifesting in enhanced secretion of IL-6. Changes in number of cells carrying ICAM-1 on their surface and activation of the synthesis of vascular endothelial growth factor during long-term exposure attest to activation of angiogenesis and inhibition of apoptosis.

Key Words: *human endothelial cells; viability; ICAM-1; interleukin-6; vascular endothelial growth factor*

The use of cultured cells as a test system makes it possible to evaluate direct effects of various components of gas mixture, including oxygen, at the cellular level. The choice of endothelial cells (EC) as the object of the study is explained by the fact that the endothelium lining all vessels in human body due to its physiology is most often exposed to changed oxygen content and hence, has mechanisms for correction of its function and adaptation to changed environmental conditions. An important role in the regulation of vasomotor reactions is played by the paracrine function of EC: synthesis of prostacyclin, NO, atrial natriuretic factor, plasminogen activator, vascular endothelial growth factor (VEGF), IL-6, and some other bioactive substances [2,3,5,19].

Acute hypoxia changes the function of EC, disorders the synthesis of proinflammatory IL, and modulates adhesion of leukocytes and platelets and permeability of the vascular wall [6]. Activation of the phosphoinositide metabolism plays an important role in these processes [20]. Long-term exposure to damaging factors, including hypoxia, leads to gradual exhaustion and distortion of the compensatory "dilating" capacity of the endothelium, and vasoconstriction and proliferation become the predominant response of EC to usual stimuli. Little is known about the effect of repeated hypoxic exposure of different duration on cultured EC.

We compared and analyzed the effects of short- and long-term repeated hypoxic exposure on the viability, expression of adhesion molecules (ICAM-1), secretion of IL-6 and VEGF by human EC in gaseous media of different composition.

MATERIALS AND METHODS

Cell culture. Primary culture of human umbilical cort EC was prepared by the routine method [12]

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with modifications (0.15% dispase was used instead of collagenase [1]). Low contamination of the resultant cultures with smooth muscle cells, no strict requirements to the duration of enzyme treatment, and the possibility of removing virtually all EC from the vascular surface result in isolation of representative cultures of the endothelium.

Cell suspension obtained after dispase treatment of the umbilical vein was centrifuged, the supernatant was discarded, the precipitate was resuspended in warm (37°C) growth medium (medium 199 with 25 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 10% FCS, 5 U/ml heparin, and 50 μg/ml endothelial cell growth factor, ECGF). The cells were inoculated in Petri dishes coated with 0.2% gelatin and incubated in a CO_2 incubator (5% CO_2, 100% humidity). The medium was replaced first after 24 h and then every 48 h, the culture were grown to confluence and then recultured. The growth and status of cultured EC were controlled under an Axiovert25 phase contrast microscope (Zeiss). Experiments were carried out with passage 4 cells. One day before the experiment, the cells were seeded at a density of 5×10^3 cell/cm^2 onto dishes coated with 0.2% gelatin and incubated under standard conditions (37°C, 5% CO_2, 100% humidity).

EIA. The concentrations of IL-6 and VEGF in culture medium after exposure in modified gaseous medium were measured by EIA on a PR2100 device (BioRad) using Human IL-6 and Human VEGF kits.

Immunophenotyping. Changes in the expression of adhesion molecules on cell surface were studied using monoclonal antibodies CD31 (PECAM-1), CD54 (ICAM-1), CD106 (VCAM-1), HLA-ABC (all from Beckman Coulter). The cells were analyzed on a Beckman Coulter Epics XL flow cytofluorometer according to manufacturer's instruction. The culture was treated with 0.25% Trypsin-EDTA, the suspension was washed, aliquots containing 2×10^5 cell/100 μl were incubated with antibodies for 15 min in darkness at 4°C.

Evaluation of culture viability. Viability of cultured EC was evaluated by fluorescent microscopy using AnnexinV-Propidium Iodide kit (Immunotech) according to the standard protocol. The method is based on simultaneous use of propidium iodide (PI) penetrating into damaged cells and reacting with DNA and AnnexinV affine for phosphatidylserine, which is located on cell surface during apoptosis and forms a specific signal for recognition of apoptotic cells. The use of the AnnexinV—PI system makes it possible to detect viable, necrotic, and apoptotic cells.

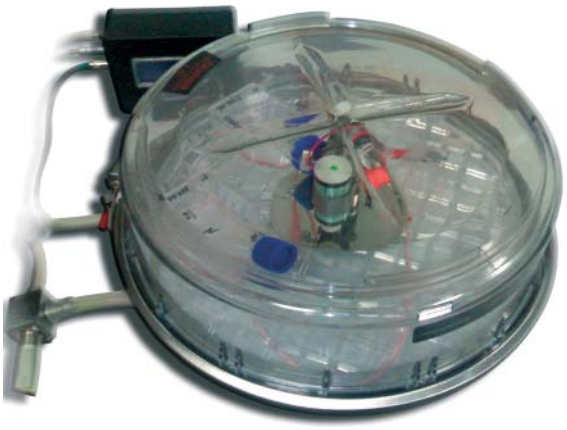

Fig. 1. Chamber for exposure of cell cultures under conditions of modified gaseous medium.

Study of endothelium migration activity on the model of mechanically damaged monolayer (wound healing). Migration activity of the endothelium was studied on a model of mechanically damaged monolayer (wound healing). Cell monolayer was damaged with a smoothened glass capillary (several rows of cells were mechanically removed). The migration was evaluated visually under a microscope and by videoimage analysis of the same visual fields before and after 4-h incubation in the test modified gas mixture. The results were processed using Mann—Whitney test.

In vitro hypoxia modeling. Exposure to modified gas composition was carried out once or repeatedly for 3 and 18 h in a sealed chamber (Stem-Cell Technology; Fig. 1). The period of normoxia in long-term exposure did not exceed 6 h. One day before the experiment, the cells were inoculated into flasks (5×10^3 cell/cm^2). After 24 h the medium was replaced and experiments were started. Flasks or Petri dishes with cells were placed into the chamber, the air was replaced with the test hypoxic mixture, and the cells were exposed under these conditions for 3 h (short-term incubations) and 18 h (long-term incubations). On the next day after a period of normoxia the experiment was repeated according to the same protocol.

Two types of mixtures with indifferent gases (argon or nitrogen) were used: 95% N_2 at 5% CO_2 and 95% Ar at 5% CO_2. Po_2 under these hypoxic conditions was 5%. Control cultures were incubated under standard conditions (37°C, 5% CO_2, 21% O_2, 100% humidity).

Analysis of images and statistical processing of the data. Videocapture and analysis of images were realized using Leica IM 1000 and Sigma Scan Pro software. The data were statistically processed using standard software.

RESULTS

Human EC used in experiments had a characteristic spindle shape and formed a dense monolayer on days 6-7 of culturing. Cells of passages 1-4 had similar morphology and proliferation rates. All cells expressed PECAM-1 molecules and ICAM-1 and VCAM-1 adhesion molecules typical of the epithelium on their surface.

For evaluation of the damaging effect of low oxygen content in the medium and compare the effects argon and nitrogen on cultured human EC we counted apoptotic and necrotic cells.

Previous data on the effects of hypoxia on human EC viability in the presence of modified Po_2 are contradictory. It was shown that short-term hypoxic exposure caused reversible selective changes in the barrier and anticoagulant functions of the endothelium and that these changes did not increase cell death [18]. On the other hand, it was shown that long-term hypoxic exposure led to an increase in vascular endothelium permeability and cell death [5,18,19].

Study of the effects of hypoxic gas mixtures showed that short-term repeated exposure in hypoxic gas mixture containing argon (gas mixture No. 1) did not increase the number of apoptotic and necrotic cells in comparison with the control (2.5±0.3% and 9.5±0.5%). A negligible increase in the percentage of necrotic cells and a reduction in the number of apoptotic cells were observed 24 and 48 h after a single 3-hour exposure. A similar trend (increase in the percentage of PI-positive cells and a decrease in the number of AnnexinV-positive cells) was observed during the reoxygenation period following double short-term exposure in the studied gas mixture. In experiments with hypoxic gas mixture containing 90% nitrogen (gas mixture No. 2) the percentage of apoptotic cells in the culture was 2% after single 3-hour exposure, increased to 4.7% after double short-term exposure, and reached 5.6% after thriple short-term exposures. The percentage of necrotic cells after exposure in gas mixture No. 2 was higher than after exposure in gas mixture No. 1: 14.4% after a single exposure, 9.3% after double exposure, and 8.4% after triple exposure. The percentage of necrotic and apoptotic cells did not change during the reoxygenation period (24 and 48 h after a single exposure) and was 13.6±0.1 and 4.7±0.5%, respectively.

Long-term incubation of cell cultures in the studied gas mixtures led to an increase in the percentage of necrotic cells in gas mixture No. 1 (by 9, 16.6, and 18% after single, double, and triple long-term exposure, respectively) without increasing the percent of apoptotically damaged cells (mean value 3%). The percentage of necrotic cells after exposure in gas mixture No. 2 was 11.8% after a single exposure and 5% after double and triple exposures. The percentage of apoptotic cells was the same 3%. The percentage of apoptotic and necrotic cells in EC culture passages under normal conditions was 2.5±0.3% and 9.5±0.5%, respectively.

Migration activity of cells is an indicator of cell function *in vitro*. Studies of migration of cultured human EC cells on a model of mechanically damaged monolayer showed that hypoxia negligibly (by no more than 15%) inhibited EC migration after short-term and long-term exposures in modified gas

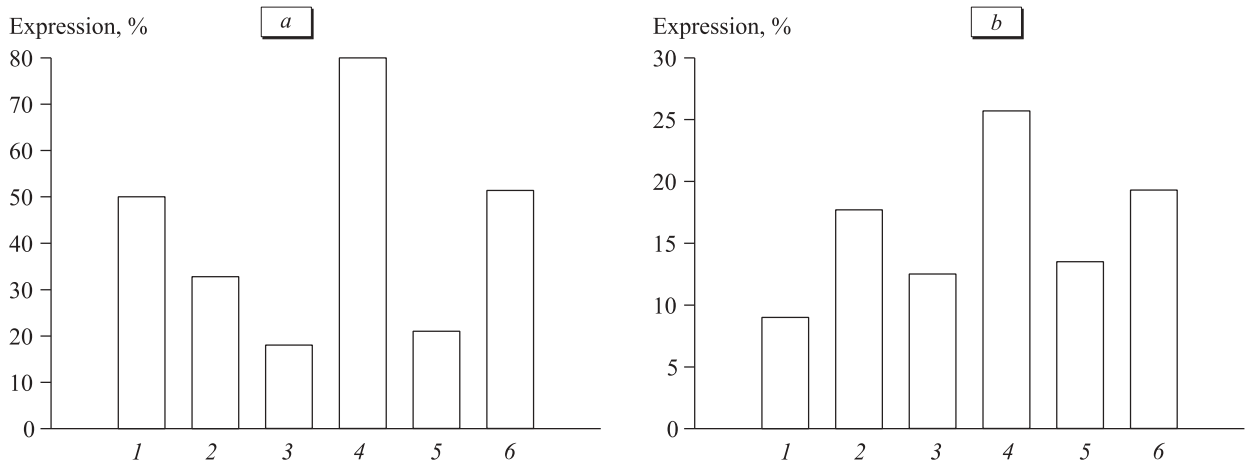

Fig. 2. Effects of short-term exposure in gas mixtures on expression of ICAM-1 by human EC. *a*) medium containing 90% N_2, 5% CO_2, and 5% O_2; *b*) medium containing 90% Ar, 5% CO_2, and 5% O_2. *1*) single 3-h exposure; *2*) single 3-h exposure and 24-h reoxygenation; *3*) single 3-h exposure and 48-h reoxygenation; *4*) double 3-h exposure; *5*) double 3-h exposure and 24-h reoxygenation; *6*) triple 3-h exposure.

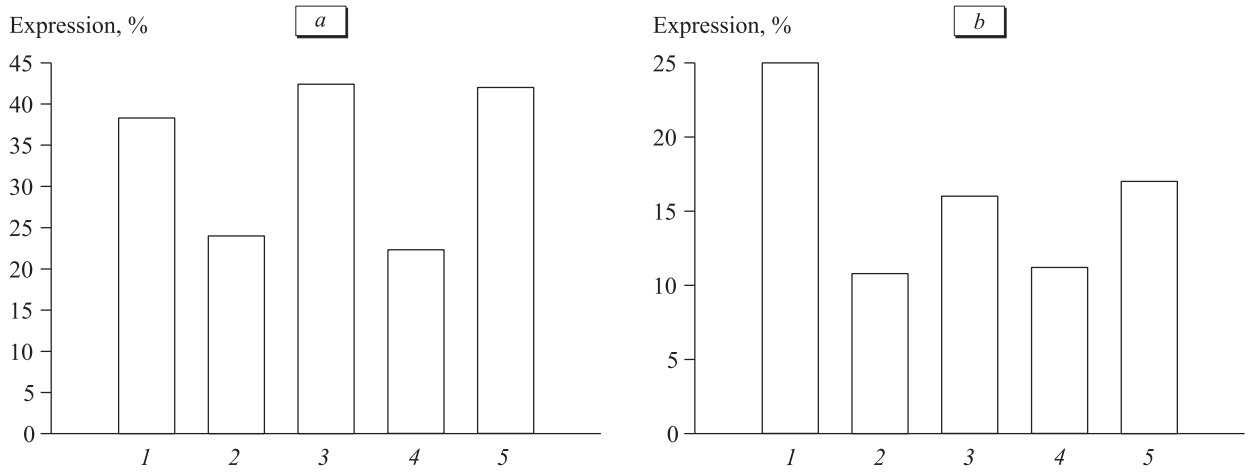

Fig. 3. Effects of long-term exposure in gas mixtures on expression of ICAM-1 by human EC. *a*) medium containing 90% N_2, 5% CO_2, and 5% O_2; *b*) medium containing 90% Ar, 5% CO_2, and 5% O_2. *1*) single 18-h exposure; *2*) single 18-h exposure and 24-h reoxygenation; *3*) single 18-h exposure and 48-h reoxygenation; *4*) double 18-h exposure; *5*) triple 18-h exposure.

media in comparison with cell culture incubated under standard conditions. No appreciable changes in cell migration in different gas mixtures were detected.

Migration activity of cells depends on adhesion characteristics of the substrate and adhesion properties of cells. A previous study of the effects of hypoxia on the expression of adhesion molecules revealed an increase in ICAM-1 expression by human EC after a single (60-min) exposure in 100% argon [7]. Activation of ICAM-1 expression on EC was observed in this experiment after treatment of the culture with IL-1 or TNF-α, increased after 6-8 h, and persisted for at least 48 h after stimulation. We showed that the percent of cells carrying this marker sharply increased (almost 3-fold in comparison with the initial level) after single exposure in hypoxic mixtures of different composition and decreased after the reoxygenation period, but did not return to the initial level (Figs. 2, 3).

Cytokine production is an important characteristic of the functional state of the cell population [3,13]. It was previously shown that long-term (18-h) exposure had no effect on IL-1 secretion: its level remained virtually unchanged (4-6 pg/ml). We also found that IL-1 synthesis remained unchanged after short-term (3 h) and long-term (18 h) repeated hypoxic exposures, its concentration in culture medium being 5-7 pg/ml [2]. This indicates that IL-1 concentration in the medium does not depend on the duration and type of exposure in hypoxic media. On the other hand, the synthesis of IL-6 by human EC during incubation in hypoxic media and reoxygenation period increased in comparison with the initial level (Figs. 2, 3). The maximum increment in IL-6 excretion was observed after the second short-term exposure to argon- and nitrogen-containing mixtures. Comparison of IL-6 production levels during the reoxygenation period showed that the concentration of this IL sharply increased after 24 h of reoxygenation and decreased after 48 and 72 h, but did not reach the control level. This time course of changes in IL-6 concentration can indicate long-term adaptation of EC to reoxygenation conditions. The secretion of IL-6 by EC increased after repeated long-term exposures, this increase being more pronounced after a single 18-h exposure (Fig. 4).

The increase in IL-6 concentration is mainly due to the effects of stress conditions on EC. It was shown that reactive oxygen forms, whose levels increased under hypoxic conditions, modulated the levels of IL-6 and VEGF synthesis [14]. The increase in the content of active oxygen forms in the cell leads to activation of the nuclear factor kB (NF-κB) and hypoxia-induced factor (HIF-1α), which activate a wide spectrum of genes responsible for cell adaptation to hypoxic conditions. The degree of increase in the synthesis of mRNA responsible for VEGF and IL-6 expression was directly proportional to the concentration of active oxygen forms, synthesized by the mitochondria during the entire period of hypoxia [13]. Moreover, no activation of genes responsible for the synthesis of IL-6 and VEGF was for a long time observed in cells with functionally deficient mitochondria. It was shown on cardiomyocytes exposed to physiological hypoxia that accumulation of active oxygen forms in the mitochondria during hypoxia reduced contractile activity of cardiomyocytes and could be corrected by addition of antioxidants into culture medium.

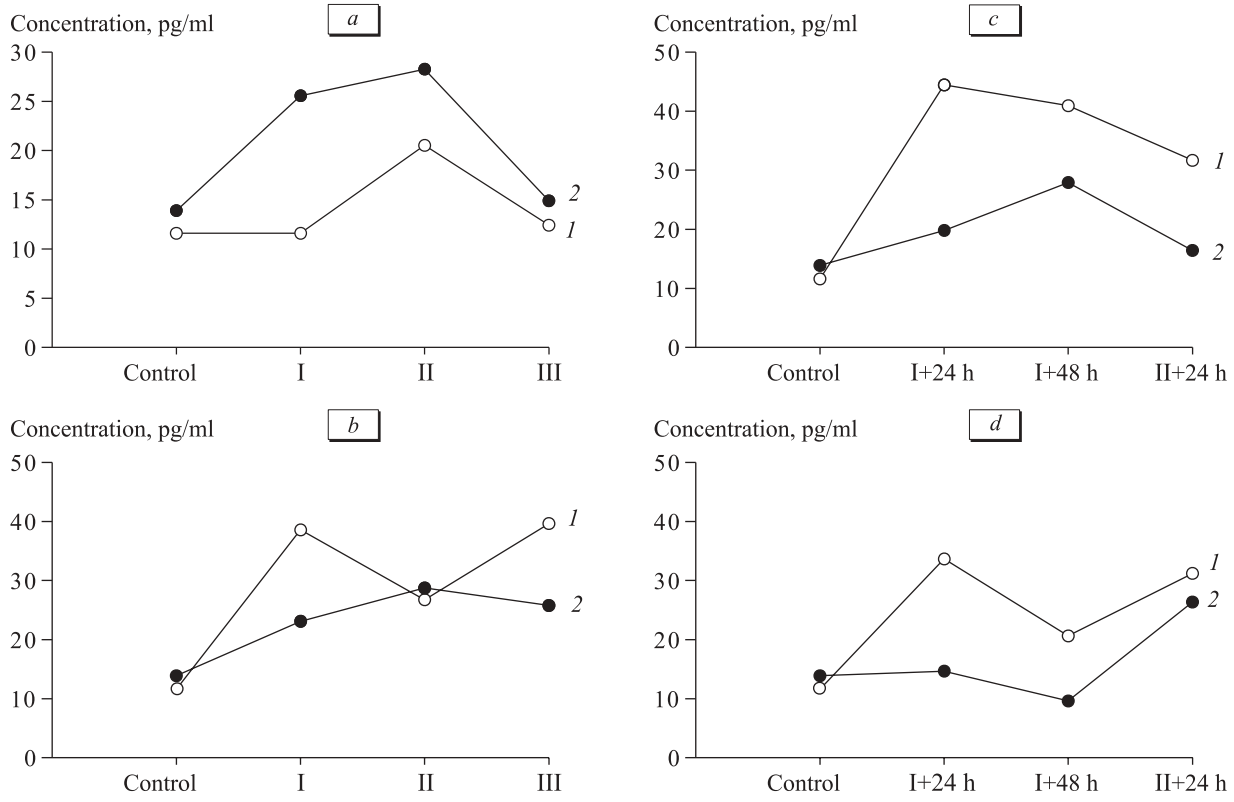

Fig. 4. Dynamics of IL-6 production by human EC after short- (*a*) and long-term (*b*) exposure to various gas mixtures and during reoxygenation after short- (*c*) and long-term (*d*) exposure. Here and on Fig. 5: *1*) medium containing 90% N_2, 5% CO_2, and 5% O_2; *2*) medium containing 90% Ar, 5% CO_2, and 5% O_2. I: single exposure; II: double exposure; III: triple exposure.

VEGF was first described as a tumor-produced agent modifying vascular permeability; later it was found to increase EC proliferation and migration during culturing [9,10,15]. The production of VEGF increases in acidosis, hypoglycemia, and hypoxia. The increase of VEGF production by the endothelium is mainly attributed to induction of HIF-1α expression in the nucleus and activation of hypoxia-sensitive genes, including VEGF. It was shown that long-term exposure of Hep3B cell culture to hypoxia (1% O_2, 48 h) increased VEGF gene expression by 10.6 times [13]. In our experiments, the level of VEGF reached 19.93 pg/ml in medium with nitrogen after a single 3-h exposure and 29.89 pg/ml in argon-containing medium *vs.* 16.45 pg/ml in the control. The levels of VEGF after double and triple exposure were 17.84 and 25.25 pg/ml in nitrogen-containing medium and 24.04 and 44.95 pg/ml in argon-containing medium, respectively. After single, double, and triple long-term exposures, VEGF level in culture medium was 39.85, 34.06, and 41.93 pg/ml in nitrogen-containing mixture and 27.57, 28.03, and 37.3 pg/ml, respectively, in argon-containing mixture. After 24-h reoxygenation following short-term hypoxic exposure, the level of VEGF first slightly increased (to 21.08 pg/ml) and then decreased, but did not reach the control level. After 18-h exposure in hypoxic mixtures of different composition, the content of VEGF increased and remained high after 24, 48, and 72 h of reoxygenation. The level of VEGF synthesis by EC exposed to argon hypoxia was somewhat higher than in the culture exposed to nitrogen hypoxia (Fig. 5).

It was previously shown that the increase of VEGF concentration in culture medium stimulated endotheliocyte migration or exhibited a protective effect and stimulated proliferation [16]. VEGF is an activator of kinases of focal adhesion. Activation of these enzymes stimulates migration of cultured human endothelium. No expected stimulation of endothelial cell migration was observed after their incubation in modified gas mixture. This effect can be explained by combined effect of IL-6 and VEGF, because it is known that these factors increase activities of MAP kinases stimulating proliferative activity of human EC.

Hence, repeated long-term hypoxic exposure does not modify the percentage of apoptotic and necrotic cells, EC migration *in vitro*, and IL-1 synthesis, but can modify the expression of markers responsible for cell-cell interactions and synthesis of bioactive substances, such as IL-6 and VEGF.

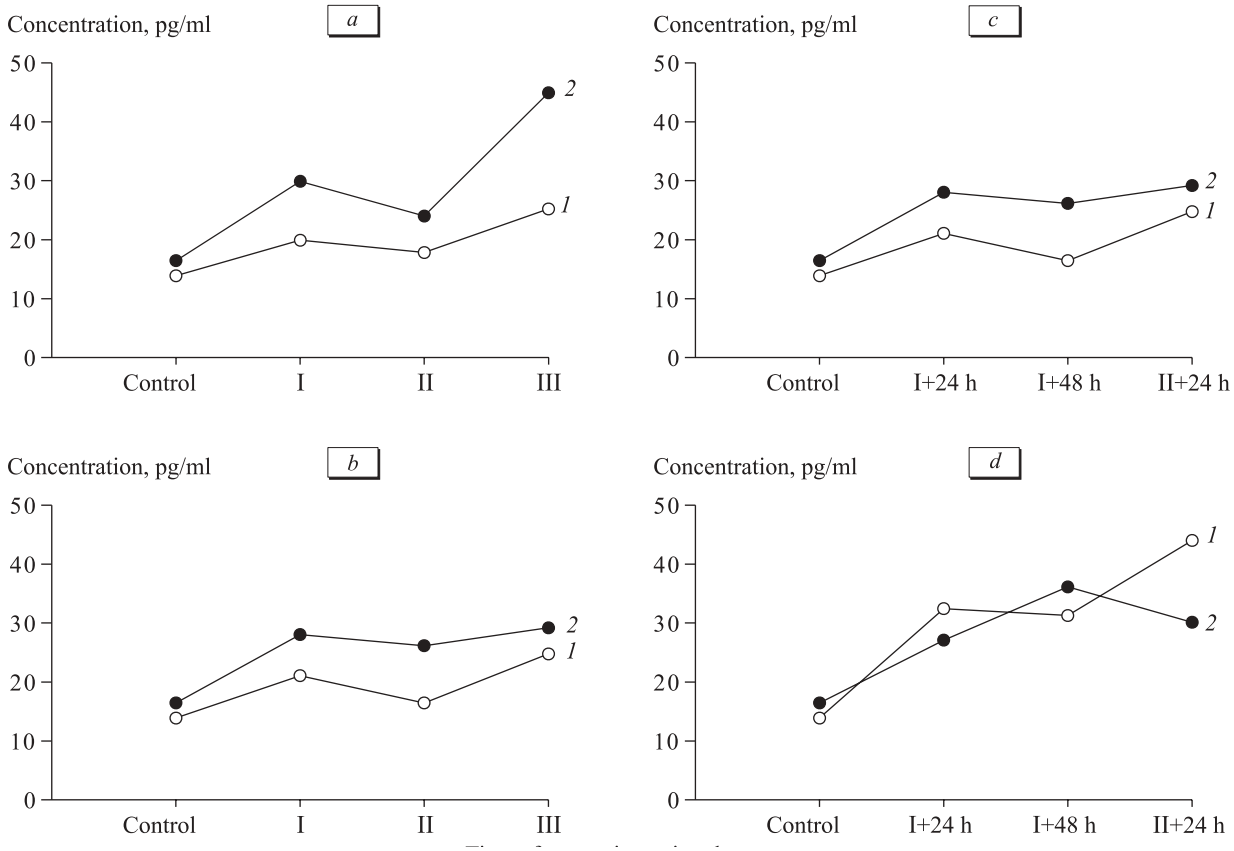

Fig. 5. Time course of VEGF synthesis by human EC after short-(*a*) and long-term (*b*) exposure to various gas mixtures and during reoxygenation after short- (*c*) and long-term (*d*) exposure.

Argon-containing mixture was less destructive for human EC, presumably due to a certain protective effect of argon under conditions of hypoxia.

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