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Activation and Damage of Endothelial Cells upon Hypoxia/Reoxygenation. Effect of Extracellular pH

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Abstract—Disturbances of blood flow upon vascular occlusions and spasms result in hypoxia and acidosis, while its subsequent restoration leads to reoxygenation and pH normalization (re-alkalization) in ischemic sites of the vascular bed. The effect of hypoxia/reoxygenation on activation and stimulation of apoptosis in cultured human endothelial cells was studied. The cells were subjected to hypoxia (2% O_2, 5% CO_2, 93% N_2) for 24 h followed by reoxygenation (21% O_2, 5% CO_2, 74% N_2) for 5 h. Reoxygenation was carried out at different pH—6.4 (preservation of acidosis after hypoxia), 7.0, and 7.4 (partial and complete re-alkalization, respectively). Hypoxia only slightly (by ~30%) increased the cell adhesion molecule ICAM-1 content on the cell surface, whereas reoxygenation more than doubled its expression. The reoxygenation effect depended on the medium acidity, and ICAM-1 increase was more pronounced at pH 7.0 compared to that at pH 6.4 and 7.4. Neither hypoxia nor reoxygenation induced expression of two other cell adhesion molecules, VCAM and E-selectin. Incubation of cells under hypoxic conditions but not reoxygenation stimulated secretion of von Willebrand factor and increased its concentration in the culture medium by more than 4 times. The percentage of cells containing apoptosis marker, activated caspase-3, was increased by approximately 1.5 times upon hypoxia as well as hypoxia/reoxygenation. Maximal values were achieved when reoxygenation was performed at pH 7.0. These data show that hypoxia/reoxygenation stimulate pro-inflammatory activation (ICAM-1 expression) and apoptosis (caspase-3 activation) of endothelial cells, and the extracellular pH influences both processes.

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Key words: endothelium, hypoxia, reoxygenation, apoptosis, cell adhesion molecules, von Willebrand factor, extracellular pH

Thrombotic occlusions and vascular spasms result in blood supply disturbances and hypoxia in damaged sites of the vascular bed. Oxygen starvation in itself causes damage [1-4] and activation [4, 5] of vascular endothelium, but a sharp increase in oxygen content (reoxygenation) after blood flow restoration (reperfusion) can also exhibit both damaging [2, 6-10] and activating [7, 11-17] effects on endothelial cells (EC). Stimulation of apoptosis in cultured cells upon hypoxia/reoxygenation has been repeatedly demonstrated using different apoptotic markers [2, 7-10]. However, results of investigations of this effect on pro-inflammatory activation of EC are contradictory. It was shown in a number of works that hypoxia/reoxygenation stimulates leukocyte adhesion to endothelium [11-13] and expression of cell adhesion molecules mediating this interaction [12-17]. But these results were not confirmed in some other studies, in which neither enhanced adhesion of white blood cells [18] nor increased content of cell adhesion molecules on the EC surface was registered under similar conditions [11, 18]. Hypoxia is known to stimulate secretion from endothelium of von Willebrand factor [4, 5], a protein involved in hemostatic and thrombotic reactions, but effects of reoxygenation on this manifestation of EC activation remain unstudied.

In the case of *in vivo* perfusion, restoration of blood flow in the ischemic part of blood vessels results in rapid change of pH from acidic values caused by hypoxia to physiological values. Similar changes in environmental

Abbreviations: EC, endothelial cell; ICAM-1, intercellular cell adhesion molecule-1; TNF, tumor necrosis factor; VCAM, vascular cell adhesion molecule.

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acidity should be considered in experiments *in vitro*, because they may contribute to endothelium activation and damage. Thus, it was shown that enhanced ICAM-1 (intercellular cell adhesion molecule-1) expression upon reoxygenation is inhibited by addition of Na^+/H^+ exchange inhibitors, preventing intracellular pH restoration in the case of re-alkalization of culture medium [14]. The Na^+/H^+ exchange inhibitors also inhibit the enhancement of agonist-induced secretion of von Willebrand factor and soluble P-selectin from EC, which is observed upon pH increase from acidic (6.4) to neutral (7.4) values [19]. However, up to the present time the effect of extracellular pH on EC under reoxygenation has not been studied.

The goal of this work was to evaluate effects of hypoxia/reoxygenation on activation and apoptosis of cultured EC at different values of extracellular pH—6.4 (retention of acidosis after hypoxia), 7.0, and 7.4 (partial and complete re-alkalization, respectively). Cell activation was registered by measuring the expression of cell adhesion molecules on the cell surface and von Willebrand factor secretion, and stimulation of apoptosis was determined by measuring the amount of activated caspase-3.

MATERIALS AND METHODS

Endothelial cell culture. Endothelial cells were obtained from human umbilical vein as described earlier [20]. Cells were grown in culture in DMEM medium with 10% fetal bovine serum, endothelium growth factor (200 μg/ml), penicillin (50 units/ml), streptomycin (50 μg/ml), and heparin (5 units/ml) (all from Gibco, USA). Experiments were carried out on confluent EC cultures of the second to fourth passages.

Hypoxia/reoxygenation. Hypoxia was created in a Heraeus incubator (USA) that enables supply of the chamber with two gases, carbon dioxide to maintain its normal concentration in the medium and nitrogen to displace oxygen. Oxygen content in gas mixture was monitored using an oxygen electrode. Before experiment EC were grown in six-well plates with 3 ml of culture medium. The scheme of experiments on effects of hypoxia and subsequent reoxygenation is shown in Fig. 1. Medium was changed 48 h before hypoxia. Immediately before beginning an experiment, half of the conditioned medium volume was taken under sterile conditions and placed at 4°C, then some of the plates were incubated under hypoxic conditions (2% O_2, 5% CO_2, 93% N_2) and others were incubated under normoxic conditions (21% O_2, 5% CO_2, 74% N_2) for 24 h. After the incubation, cultivation medium was replaced by the earlier taken conditioned medium with pH adjusted in advance to 6.4, 7.0, or 7.4. Then all plates were incubated under normoxia (reoxygenation) for 5 h (in most experiments). During the experiments, the pH was repeatedly measured after cell cultivation both under hypoxia and normoxia.

Stimulation of endothelial cell activation and apoptosis by tumor necrosis factor and staurosporine. A portion of cells was cultivated under normoxic conditions for 24 h in the presence of tumor necrosis factor (TNF; Sigma, USA; 100 units/ml) inducing expression of EC cell adhesion molecules, whereas another portion of cells was incubated for 5 h also under normoxic conditions in the presence of 50 nM apoptosis inducer staurosporine (Sigma).

Flow cytofluorimetry. Flow cytofluorimetry was used for determination of cell adhesion molecule (ICAM-1, VCAM (vascular cell adhesion molecule), and E-selectin) amounts on EC surface as well as for detection of intracellular apoptosis marker activated caspase-3. For all determinations, cells were analyzed using a FACS Calibur flow cytofluorimeter (Becton Dickinson, USA). At least 5000 cells were analyzed in each sample (in most samples – 10,000 cells).

Cells were suspended in trypsin/EDTA solution (0.02%/0.05%), placed on ice, and prefixed with 0.02% paraformaldehyde solution for 1-2 min. The fixative was removed by washing, and after resuspension cells were

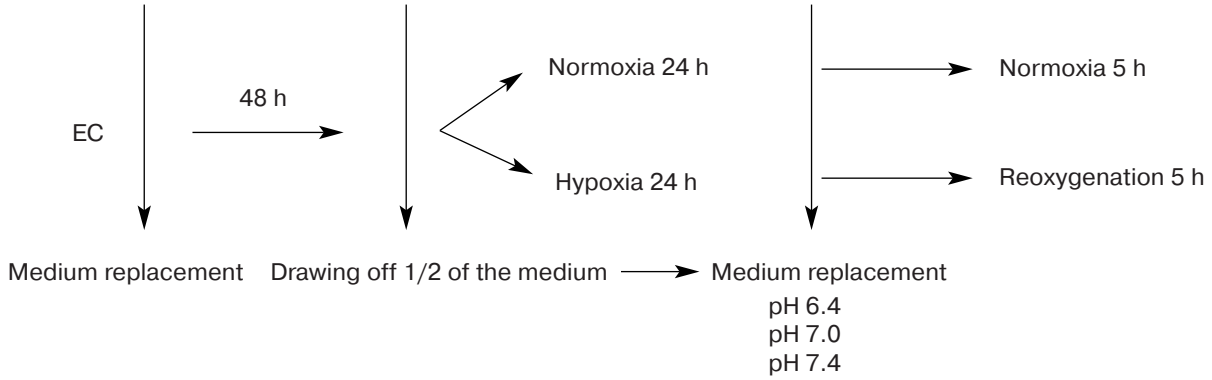

Fig. 1. Scheme of an experiment on the effect of hypoxia/reoxygenation on endothelial cells in culture (explanations in the text).

divided to three parts in each analyzed population for following determination of (i) ICAM-1, (ii) VCAM and E-selectin, and (iii) activated caspase-3. For the ICAM-1 determination, biotinylated monoclonal antibodies 10F3B2 were used [21] at the concentration of 10 μg/ml (antibodies were the courtesy of M. M. Peklo, Russian Cardiology Research and Production Complex) and then after washing off the antibodies, phycoerythrin (PE)-labeled streptavidin (Caltag, USA) was added. Antibodies against ICAM-1 were not added into control samples (nonspecific cell fluorescence). For VCAM and E-selectin analysis, commercial antibodies CD106-FITC and CD62E-PE, respectively, were used (both antibodies of BD Pharmingen, USA) at the concentration recommended by the producer (20 μl per 10^6 cells). Control samples were incubated without antibodies. All incubations were carried out on ice for 30 min, and phosphate buffered saline containing 2% BSA was used for intermediate washings and cell resuspension. After the last incubation, cells were washed and resuspended in 50 μl buffer and then fixed by addition of 300 μl 2% paraformaldehyde solution. Fixed cells were stored no more than 24 h in the dark at 4°C before analysis. Mean fluorescence values for the cell distribution histograms were determined for all samples. The fluorescence level of cells incubated for 24 h under normoxia conditions was taken as 100%.

Activated caspase-3 within EC was determined using the Active Caspase-3 FITC Mab Apoptosis Kit I (BD Pharmingen) according to the manufacturer's protocol. Cell suspension was pre-fixed with paraformaldehyde (see above) and treated with a special permeabilization and fixative solution (BD Cytofix/Cytoperm^{TM}) and then stained with the FITC-labeled antibodies against activated caspase-3. The percentage of positively stained cells, i.e. those containing activated caspase-3 ((+) cells), was determined in each analysis using cells without added labeled antibodies as a negative control.

Determination of von Willebrand factor concentration. Von Willebrand factor was determined in the EC cultivation medium by immunoenzyme analysis according to a previously described technique [22].

Statistics. Statistical analysis was carried out using the Statistica 6.0 program. The analyzed parameters had abnormal distribution (Shapiro–Wilks test), and significance of differences between groups of dependent variables (effects on variables in the same EC culture) was determined using non-parametric statistics. The paired Wilkoxon test was used for comparison of two groups (hypoxia and reoxygenation effects comparing to that of normoxia), whereas comparison of several groups (effects of different pH) was performed by Freidman ANOVA, followed by determination of significance of differences between separate groups using Kruskal–Wallis test. Data are presented as means ± standard errors of means for $n \geq 4$ and as means for $n < 4$.

RESULTS

In this work we have studied the effect of EC hypoxia and following reoxygenation on (i) expression of cell adhesion molecules (ICAM-1, VCAM, and E-selectin) on the cell surface, (ii) secretion of von Willebrand factor, and (iii) intracellular content of apoptosis marker, activated caspase-3. Cells were incubated under hypoxic conditions for 24 h and then, after change of cultivation medium, under normoxia (reoxygenation) for 5 h (in most experiments). Reoxygenation effects were investigated at different pH values of the cultivation medium—6.4, 7.0, and 7.4. The value pH 6.4 approximately corresponds to the level of medium acidity after hypoxia, while pH 7.0 and 7.4 correspond to its partial and complete realkalization, respectively. Control cells were incubated at normal oxygen content for identical periods of time and in the same media as cells after hypoxia (see also Fig. 1).

Endothelial cells constitutively express on their surface the cell adhesion molecule ICAM-1 (the average level of cell fluorescence under normoxia is 14.4 ± 2.9 with nonspecific fluorescence 2.2 ± 0.2). The ICAM-1 content on EC surface was slightly but significantly (approximately by 30%) increased after cell incubation under hypoxia. At the same time, subsequent action of hypoxia and reoxygenation almost doubled ICAM-1 expression compared to cells incubated only under normal conditions (Table 1 and Fig. 2). Investigation of dynamics of the ICAM-1 expression during reoxygenation has shown that maximal levels were achieved at 6 h after the end of hypoxic incubation (Fig. 3). Because of this, most experiments on investigation of reoxygenation effects were carried out using the 5-h incubation. The reoxygenation effect depends on pH of the culture medium. At pH 7.0, the ICAM-1 content was significantly higher than at pH 6.4 and 7.4 (Fig. 4).

Non-activated endothelial cells contain practically no VCAM and E-selectin cell adhesion molecules on their surface (the mean level of cell fluorescence under normoxic conditions – 3.8 ± 0.5 and 4.1 ± 0.7, while nonspecific fluorescence – 2.1 ± 0.5 and 2.0 ± 0.4, respectively). Neither hypoxia nor following reoxygenation induced expression of these molecules (Table 1). Reoxygenation at different pH of the culture medium also had no effect either on VCAM content (125, 125, and 131% of normoxia level at pH 6.4, 7.0, and 7.4, respectively, mean values, $n = 3$) or E-selectin (103, 108, and 114% of normoxia level at pH 6.4, 7.0, and 7.4, respectively, mean values, $n = 3$).

Incubation of cells in the presence of TNF, a classical inducer of expression of most cell adhesion molecules, used as positive control, resulted in significant (at least several-fold) increase of all three molecules under study (Table 1; for ICAM-1 expression see also Fig. 2).

Hypoxia also stimulated von Willebrand factor secretion from EC, increasing the amount of this protein in the culture medium by more than fourfold compared to

normoxia. However, the following reoxygenation did not result in its increased accumulation; its concentration was identical in culture medium of cells earlier affected by hypoxia and incubated under normal conditions only (Table 2). Changes in medium acidity during reoxygenation also had no effect on the content of von Willebrand factor (304 ± 111, 246 ± 88, 225 ± 90 ng/ml at pH 6.4, 7.0, and 7.4, respectively, means ± errors of means, $n = 5$, differences are not significant).

Stimulation of EC apoptosis was assessed by measuring the content of activated caspase-3, the key enzyme of apoptotic cell damage. The percentage of the activated caspase-containing cells was increased by approximately 1.5-fold after hypoxia and hypoxia/reoxygenation compared to cultures incubated under normoxic conditions (Table 3 and Fig. 5). Like in the case of ICAM-1 expression, maximal values were registered at pH 7.0 (Fig. 6). In this series of experiments the classical apoptosis inducer staurosporine (nonspecific protein kinase inhibitor) was used as positive control, and it increased more than threefold the number of cells containing activated caspase-3 (Table 3 and Fig. 5).

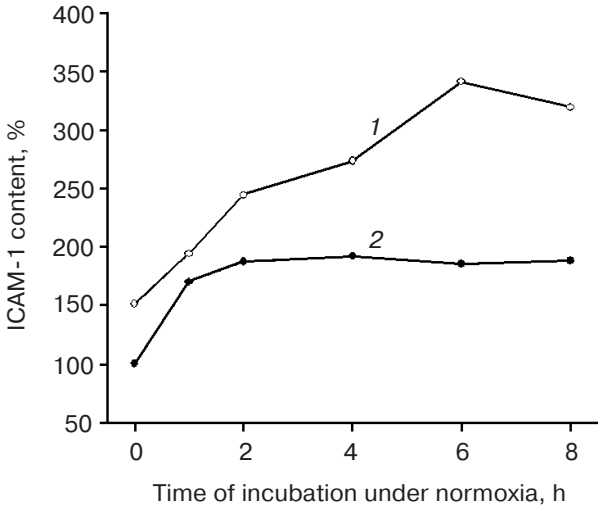

Fig. 3. Dynamics of ICAM-1 expression on EC surface during reoxygenation: *1*) after hypoxia (reoxygenation) (cells were preincubated under hypoxia, then culture medium was changed (pH 7.0) and cells were incubated under normoxia for indicated time); *2*) without hypoxia (cells were preincubated under normoxia for 24 h, then culture medium was changed (pH 7.0) and incubation continued under normoxia for indicated time). The level of ICAM-1 expression after 24 h preincubation under normoxia was taken as 100%. Means of three experiments are shown.

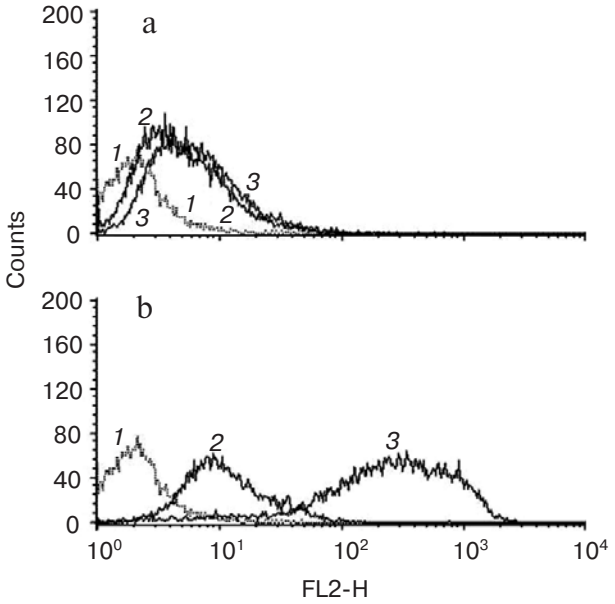

Fig. 2. ICAM-1 determination on EC surface. Flow cytofluorimetry distribution histogram. Fixed EC were incubated with biotinylated antibody 10F3B2 against ICAM-1 and then with the PE-labeled streptavidin. a) Histograms: *1*) negative control (cells grown for 24 h in culture under normoxia (24 h) were stained only by PE-streptavidin without antibody 10F3B2); *2*) cells incubated under normoxia for 24 h; *3*) cells incubated under hypoxia for 24 h. b) Histograms: *1*) negative control (see above); *2*) cells incubated under hypoxia for 24 h and then for 5 h under normoxia at pH 7.0 (reoxygenation); *3*) cells incubated under normoxia for 24 h in the presence of 100 units/ml TNF. The PE (FL2-H) fluorescence is shown on the abscissa axis, and number of cells (Counts) is shown on the ordinate axis.

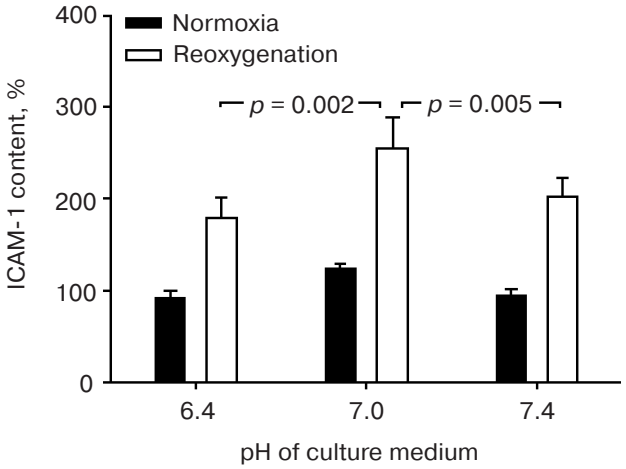

Fig. 4. Effect of culture medium pH on ICAM-1 expression during reoxygenation. White columns: cells were preincubated under hypoxia, medium was replaced by another one with indicated pH, and incubation continued under normoxia (reoxygenation) for 5 h. Black columns: cells were preincubated under normoxia for 24 h, medium was replaced by another one with indicated pH, and incubation continued under normoxia (normoxia) for 5 h. The level of ICAM-1 expression after 24 h preliminary incubation under normoxia was taken as 100%. Means ± errors of means from five experiments are shown. The ICAM-1 level increase under reoxygenation comparing to that under normoxia is significant at all pH values ($p < 0.05$). Significances of differences between ICAM-1 levels under reoxygenation at pH 7.0 and pH 6.4 and 7.4 are presented in the figure. Differences between pH 6.4 and 7.4 are not significant.

Table 1. Endothelial adhesion molecules upon hypoxia/reoxygenation

Incubation conditions	ICAM-1 ($n = 18$)	VCAM ($n = 5$)	E-selectin ($n = 5$)
	Content, % of normoxia 24 h		
Normoxia 24 h	100	100	100
Hypoxia 24 h	139 ± 12; $p = 0.015$[1]	118 ± 17; n.s.[1]	123 ± 5; n.s.[1]
Normoxia 24 h + normoxia 5 h (pH 7.0)	134 ± 14	119 ± 16	130 ± 33
Hypoxia 24 h + reoxygenation 5 h (pH 7.0)	281 ± 28; $p < 0.001$[2]	112 ± 14; n.s.[2]	100 ± 7; n.s.[2]
Normoxia 24 h + TNF	5530 ± 1441; $p < 0.001$[1]	389 ± 174; $p = 0.043$[1]	439 ± 91; $p = 0.043$[1]

Note: Data are presented as percent of the content of cell adhesion molecules on the EC surface after incubation for 24 h under normoxia (100%). Reoxygenation was carried out at pH 7.0. n is number of experiments. Means ± errors of means are shown. [1] p, significance of differences from normoxia 24 h; [2] p, significance of differences from normoxia 24 h + normoxia 5 h (pH 7.0); n.s., not significant.

Table 2. Von Willebrand factor in endothelial cell culture medium upon hypoxia/reoxygenation

Incubation conditions	Von Willebrand factor, ng/ml ($n = 12$)
Normoxia 24 h	211 ± 19
Hypoxia 24 h	872 ± 148; $p = 0.002$[1]
Normoxia 24 h + normoxia 5 h (pH 7.0)	199 ± 30
Hypoxia 24 h + reoxygenation 5 h (pH 7.0)	231 ± 35; n.s.[2]

Note: Von Willebrand factor was determined in the EC culture medium after incubation under corresponding conditions. Reoxygenation was carried out at pH 7.0. n is number of experiments. Means ± errors of means are shown. [1] p, significance of differences from normoxia 24 h; [2] p, significance of differences from normoxia 24 h + normoxia 5 h (pH 7.0); n.s., not significant.

Table 3. Activated caspase-3 in endothelial cells upon hypoxia/reoxygenation

Incubation conditions	Activated caspase-3, % of (+) cells ($n = 6$)
Normoxia 24 h	5.9 ± 1.3
Hypoxia 24 h	9.0 ± 2.0; $p = 0.046$[1]
Normoxia 24 h + normoxia 5 h (pH 7.0)	6.9 ± 0.9
Hypoxia 24 h + reoxygenation 5 h (pH 7.0)	10.9 ± 1.4; $p = 0.028$[2]
Normoxia 24 h + normoxia 5 h (pH 7.0), staurosporine	24.1 ± 3.0; $p = 0.028$[2]

Note: Percentage of cells containing activated caspase-3 ((+) cells) was determined after incubation under corresponding conditions. Reoxygenation was carried out at pH 7.0. n is a number of experiments. Means ± errors of means are shown. [1] p, significance of differences from normoxia 24 h; [2] p, significance of differences from normoxia 24 h + normoxia 5 h (pH 7.0).

DISCUSSION

Effects of hypoxia and following reoxygenation on activation and apoptosis of human EC in culture were studied in this work. Hypoxia only slightly increased the content of cell adhesion molecule ICAM-1 on the cell surface, but subsequent reoxygenation resulted in more pronounced increase in expression of this protein. Similar results were obtained in most previous works, which also indicated stimulation of ICAM-1 expression after hypoxia/reoxygenation [12, 14-17]. Earlier we could not reveal significant increase in the ICAM-1 content after cell incubation only under hypoxia conditions [4]. However, in this work these distinctions reached the significant level due to a large number of experiments ($n = 18$). Nevertheless, approximately 30% growth of ICAM-1 in hypoxic conditions was significantly lower than the twofold increase after following reoxygenation. The fact that reoxygenation is the main stimulus for ICAM-1 increase also agrees with results of other authors [15, 17]. It should be noted that similar results were obtained, although in one work [15] cells were incubated for a short period of time (60 min) under complete anoxia, and in another [17], like in our study, for almost 24 h at oxygen content of 1-2%. Unlike ICAM-1, a protein constantly present on the EC surface, other two cell adhesion molecules under study, E-selectin and VCAM, are either not expressed or are present in an insignificant amount on

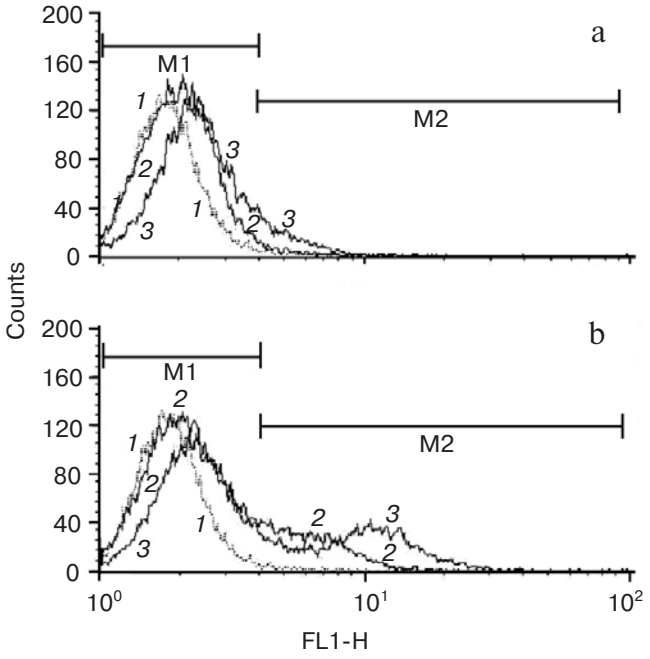

Fig. 5. Detection of EC containing activated caspase-3 ((+) cells. Flow cytofluorimetry distribution histograms. EC treated by permeabilization and fixation buffer were incubated with FITC-labeled antibody against activated caspase-3. a) Histograms: *1*) negative control (cells grown in culture for 24 h under normoxia were analyzed without addition of FITC-labeled antibody); *2*) cells were incubated under normoxia for 24 h; *3*) cells were incubated under hypoxia for 24 h. b) Histograms: *1*) negative control (see above); *2*) cells were incubated under hypoxia for 24 h, then under normoxia for 5 h at pH 7.0 (reoxygenation); *3*) cells were incubated under normoxia for 24 h and then for 5 h (pH 7.0) in the presence of 50 nM staurosporine. The abscissa axis shows FITC (FL1-H) fluorescence, ordinate axis shows the number of cells (Counts). M1 and M2 are histogram regions corresponding to caspase-3 (–) and (+) cells.

non-activated EC. We did not register induction of expression of these molecules either in response to hypoxia or to following reoxygenation. Earlier investigations of E-selectin and VCAM expression under hypoxia/reoxygenation are not as numerous as in the case of ICAM-1, and their results are rather contradictory. There are separate works showing both the absence of E-selectin [11, 18] and VCAM [18] induction and the increased content, respectively, of the first [12] and second [13, 17] proteins under these conditions. These contradictions are probably explained by differences in protocols of experiments, first of all by the depth and duration of hypoxia (from 30-60 min under complete anoxia to 24 h at oxygen content of 1-2%), reoxygenation conditions (medium exchange or its retention after hypoxia, the length of incubation), and features of the EC used (source, passage, etc.). Combination of the data from investigation of cell adhesion molecules suggests that among proteins mediating endothelium interactions with leukocytes, ICAM-1 is the most sensitive and reproducible marker of the EC pro-inflammatory activation induced by hypoxia/reoxygenation.

Results obtained in this work confirm our previous data [4] and those of other authors [5] showing that hypoxia stimulates von Willebrand factor secretion from EC. We have also shown for the first time that reoxygenation does not increase the content of this protein in the culture medium compared to cells incubated only under normoxia. These data indicate that, unlike increase of ICAM-1 expression, endothelium activation exhibited in the von Willebrand factor secretion is induced by hypoxia rather than by reoxygenation.

The percentage of cells containing activated caspase-3 served as a marker of apoptotic damage of EC. It was shown that hypoxia increases the number of such cells, while reoxygenation neither stimulates its further increase nor causes a decrease in their number, which reached approximately 10% of the whole population in response to these effects. These results mainly agree with earlier data of other authors. The use of different methods of apoptosis estimation (activation of caspase-3 and caspase-9, annexin V binding, DNA fragmentation, etc.) made it possible to show that the emergence of apoptotically damaged cells was due to both hypoxia and following reoxygenation. The number of these cells varied in different works from 5% to approximately 25% [2, 7-10].

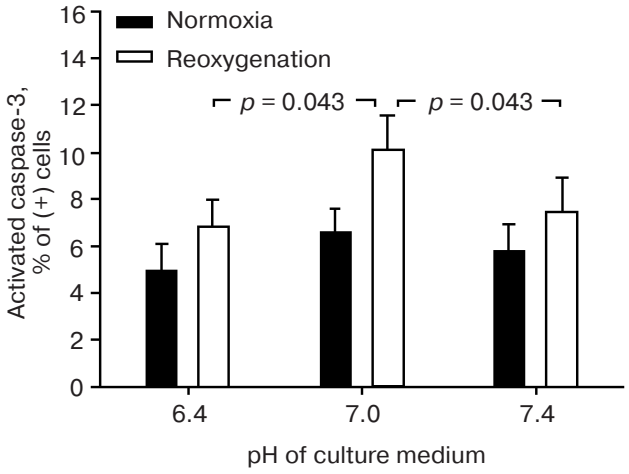

Fig. 6. Effect of culture medium pH on percentage of the activated caspase-3-containing EC ((+) cells) upon reoxygenation. White columns: cells were preincubated under hypoxia, medium was replaced by another one with indicated pH, and incubation continued under normoxia (reoxygenation) for 5 h. Black columns: cells were preincubated under normoxia for 24 h, medium was replaced by another one with indicated pH and incubated again under normoxia (normoxia) for 5 h. Means ± errors of means from five experiments are shown. The increase in percentage of (+) cells upon reoxygenation compared to that under normoxia is significant at all pH values ($p < 0.05$). Significance of differences between percentage of (+) cells upon reoxygenation at pH 7.0 and pH 6.4 and 7.4, respectively, is shown in the figure. Differences at pH 6.4 and 7.4 are not significant.

Yu et al. [9] also used activated caspase-3 as a marker of apoptosis, and in their study, like in our work, the number of cells containing active enzyme was about 10%. Zhao et al. [8] measured the caspase-3 enzyme activity by a colorimetric test, and they registered after cell incubation under hypoxia/reoxygenation approximately 1.5-fold increase in enzyme activity, which is also in good agreement with our data. It should be noted that in some works, unlike this one, damaging effect of reoxygenation exceeded that of hypoxia [2, 7, 8]. We suppose that, like in the case of expression of cell adhesion molecules, such variations are first of all the result of different experimental conditions (see above).

Hypoxia both *in vitro* and *in vivo* is accompanied by acidification of the extracellular medium, whereas following reoxygenation is accompanied by re-alkalization, i.e. pH restoration to normal physiological values. These effects are usually not considered in experiments on reoxygenation effects on different cells, including endothelial cells. In this work, we have studied for the first time parameters of EC activation and apoptosis using media with different pH (6.4, 7.0, and 7.4) in experiments on reoxygenation. In all cases, we used conditioned medium previously incubated with cells under normal conditions, because addition of fresh medium to the cells is already a stress effect and may result in alteration of their functioning. The pH value 6.4 was chosen as the average index of medium acidity after cell cultivation under hypoxia. Two other pH values, 7.0 and 7.4, were chosen as parameters that can be achieved under partial and complete re-alkalization, respectively. Besides, in choosing pH 7.0 we have taken into account that this value approximately corresponds to parameters of the medium acidity after standard EC cultivation for 1-2 days. Similar pH values are also established in the case of reoxygenation without medium exchange after hypoxia. We found reliable distinctions in the level of ICAM-1 expression and in the percentage of cells containing activated caspase-3 after reoxygenation depending on pH. Maximal values of both parameters were achieved at pH 7.0. These results are indicative of the influence of the medium acidity on the EC activation and apoptosis parameters upon reoxygenation and necessity of pH control in such experiments. It is known that moderate acidification of the environment and corresponding decrease in intracellular pH upon hypoxia is able to protect cells against damaging effect of ischemia, whereas following restoration of normal pH values upon reperfusion and reoxygenation, in contrast, can enhance their damage [23, 24]. This phenomenon was called the "pH paradox" and was confirmed in experiments using the Na^+/H^+ exchange inhibitors preventing the cell cytoplasm re-alkalization upon increase in extracellular pH. In particular, it was shown that the use of these compounds improves survival of cardiomyocytes upon reperfusion after ischemia [24]. It was shown in experiments on EC that the Na^+/H^+ exchange inhibitors inhibit ICAM-1 expression stimulated by reoxygenation [14]. Our data also show that preservation of moderate acidosis (pH 6.4) after hypoxia decreases the EC activation and apoptosis upon subsequent reoxygenation. However, it is still not clear why maximal levels of these reactions were registered at pH 7.0 but not at pH 7.4, i.e. at partial rather than at complete re-alkalization of the culture medium.

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REFERENCES

1. Stempien-Otero, A., Karsan, A., Cornejo, C. J., Xiang, H., Eunson, T., Morrison, R. S., Kay, M., and Harlan, J. (1999) *J. Biol. Chem.*, **274**, 8039-8045.
2. Li, D., Yang, B., and Mehta, J. L. (1999) *Cardiovasc. Res.*, **42**, 805-813.
3. Lee, C. N., Cheng, W. F., Chang, M. C., Su, Y. N., Chen, C. A., and Hsieh, F. J. (2005) *Apoptosis*, **10**, 887-894.
4. Antonova, O. A., Loktionova, S. A., Golubeva, N. V., Romanov, Yu. A., and Mazurov, A. V. (2007) *Byul. Eksp. Biol. Med.*, **10**, 384-386.
5. Pinsky, D. J., Naka, Y., Liao, H., Oz, M. C., Wagner, D. D., Mayadas, T. N., Johnson, R. C., Hynes, R. O., Health, M., Lawson, C. A., and Stern, D. M. (1996) *J. Clin. Invest.*, **97**, 493-500.
6. Scarabelli, T. M., Stephanou, A., Rayment, N., Pasini, E., Comini, L., Curello, S., Ferrari, R., Knight, R., and Latchman, D. S. (2001) *Circulation*, **104**, 253-256.
7. Mold, C., and Morris, C. A. (2001) *Immunology*, **102**, 359-364.
8. Zhao, H., Miller, M., Pfieffer, K., Buras, J. A., and Stalh, G. L. (2003) *FASEB J.*, **17**, 723-734.
9. Yu, E. Z., Li, Y. Y., Liu, X. H., Kagan, E., and McCarron, R. M. (2004) *Lab. Invest.*, **84**, 553-561.
10. Bresgen, N., Kahlhuber, G., Krizbai, I., Bauer, H., Bauer, H. C., and Eckl, P. M. (2003) *J. Neurosci. Res.*, **72**, 327-333.
11. Yoshida, N., Granger, D. N., Anderson, D. C., Pothlein, R., Lane, C., and Kvietys, P. R. (1992) *Am. J. Physiol.*, **262**, H1891-H1898.
12. Ichikawa, H., Flores, S., Kvietys, P. R., Wolf, R. E., Yoshikawa, T., Granger, D. N., and Aw, T. Y. (1997) *Circ. Res.*, **81**, 922-931.
13. Kokura, S., Wolf, R. E., Yoshikawa, T., Ichikawa, H., Granger, D. N., and Aw, T. Y. (2000) *Microcirculation*, **7**, 13-23.
14. Hattori, R., Otani, H., Moriguchi, Y., Matsubara, H., Yamamura, T., Nakao, Y., Omiya, H., Osako, M., and Imamura, H. (2001) *Am. J. Physiol. Heart Circ. Physiol.*, **280**, H2796-H2803.
15. Ziegelstein, R. C., He, C., and Hu, Q. (2004) *Biochem. Biophys. Res. Commun.*, **10**, 68-73.
16. Lan, W., Harmon, D., Wang, J. H., Shorten, G., and Redmond, P. (2004) *Eur. J. Anaesthesiol.*, **21**, 967-972.

17. Corcoran, T. B., Engel, A., and Shorten, G. D. (2006) *Eur. J. Anaesthesiol.*, **23**, 942-947.
18. Maurus, C. F., Schmidt, D., Schneider, M. K., Nurina, M. I., Seebach, J. D., and Zund, G. (2003) *Eur. J. Cardiothorac. Surg.*, **23**, 976-983.
19. Huck, V., Niemeyer, A., Goerge, T., Schaeker, E. M., Ossig, R., Rogge, P., Schneider, M. F., Oberleithner, H., and Schneider, S. W. (2007) *J. Cell. Physiol.*, **211**, 399-409.
20. Antonov, A. S., Nikolaeva, M. A., Klueva, T. S., Romanov, Y. A., Babaev, V. R., Bystrevskaya, V. B., Perov, N. A., Repin, V. S., and Smirnov, V. N. (1986) *Atherosclerosis*, **59**, 1-19.
21. Printseva, O. Yu., Peclo, M. M., and Grown, A. M. (1992) *Am. J. Pathol.*, **140**, 889-896.
22. Yanushevskaya, E. V., Barkevich, E. A., Khaspekova, S. G., Naymushin, Ya. A., Vlasik, T. N., Likhacheva, E. A., Plyushch, O. P., and Mazurov, A. V. (2005) *Gematol. Transfuziol.*, **50**, 20-25.
23. Kaplan, S. H., Yang, H., Gilliam, D. E., Shen, J., Lemasters, J. J., and Cascio, W. E. (1995) *Cardiovasc. Res.*, **29**, 231-238.
24. Lemasters, J. J., Bond, J. M., Chacon, E., Harper, I. S., Kaplan, S. H., Ohata, H., Trollinger, D. R., Herman, B., and Cascio, W. E. (1996) *EXS*, **76**, 99-114.