2. M. A. Flerov, A. E. Borovitskaya, and T. I. Tolstukhina, The Nervous System [in Russian], Vol. 21, Leningrad (1980).
3. E. Z. Lmirbekov and S. P. L'vova, Mechanisms of Biochemical Changes at Low Body Temperatures [in Russian], Rostov-on-Don (1985)
4. E. G. Gray and V. P. Whittaker, J. Anat., **96**, 79 (1962).
5. O. H. Lowry and J. J. Lopes, J. Biol. Chem., **162**, 421 (1946).
6. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, J. Biol. Chem., **193**, 265 (1951).

EFFECTS OF HYPOXIA ON PHOSPHOINOSITIDE METABOLISM AND THE ADENYLATE CYCLASE SYSTEM IN ENDOTHELIAL CELL CULTURES

L. B. Buravkova, T. Yu. Mirzapoyazova, G. Yu. Grigoryan, and V. A. Tkachuk

UDC 616-018.74-008.931:577.152.633]-02:
616-008.922.1-008.64]-092.4

KEY WORDS: endothelial cultures, hypoxia, phosphoinositide metabolism, adenylate cyclase system, histamine

The fact is not in dispute that vascular tone is regulated through the direct participation of endothelial cells (EC). The endothelium plays a particularly important role in maintenance of the necessary level of the circulation in vitally important organs when the body is in a state of oxygen insufficiency [6, 8]. The possible existence of pO_2-chemoreceptors in the EC of arteries has been discussed [3]. However, the problem of the mechanism of changes taking place in EC during hypoxia remains unclear. This is because of difficulties in differentiation of reactions arising in EC under the influence of a low pO_2 from the effects of the large quantity of mediators circulating in the blood in hypoxia. There is thus a need for the investigations of regulatory processes during hypoxia in endothelial cell cultures, which preserve their basic properties during passage [8]. The key factor in the regulation of cell metabolism is the system of secondary messengers, responsible for transmitting a broad spectrum of external signals through the cell membrane [1, 2]. An important place among them is occupied by the adenylate cyclase complex and phosphoinositide (PI) metabolism. The active metabolites of these complex regulatory systems are cyclic 3′,5′-adenosine monophosphate (cAMP), inositol-1,4,5-triphosphate (IP_3), and diacylglycerol (DAG). IP_3 is known to intensify the release of Ca^{2+} from the endoplasmic reticulum, whereas DAG activates protein kinase C (PKC), which phosphorylates a large number of structural and functional proteins, modifying cellular activity [2, 4].

The effect of hypoxia and of Ca-mobilizing hormones on the system of secondary messengers was investigated in cultured EC from human blood vessels.

EXPERIMENTAL METHOD

EC were isolated from the human umbilical vein by perfusion of the vessel with 0.1% collagenase, and were cultured in medium 199 with Earle's salts ("Flow Lab," Great Britain), with 20% human serum, endothelial growth factor from human brain (200 μ/ml), and with heparin (100 μ/ml) [9]. Membrane fractions of EC were isolated from the confluent cell layer after the 2nd-5th passage. The cells were resuspended in buffer containing 10 mM HEPES, 150 mM NaCl, 1 mM EDTA, pH 7.5, at 4°C. The cells were then frozen in liquid nitrogen, thawed, homogenized with a ground glass homogeniz-

Institute for Medico-Biological Problems, Ministry of Health of the USSR. Institute of Experimental Cardiology. All-Union Cardiologic Scientific Center, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR A. V. Smol'yannikov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 111, No. 5, pp. 464-466, May, 1991. Original article submitted July 5, 1991.

 0007-4888/91/0005-0594$12.50

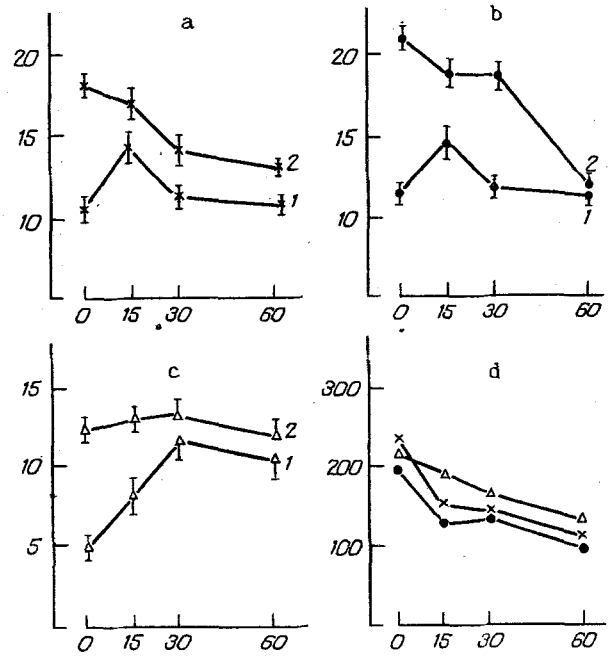

Fig. 1. Intensity of PI metabolism in EC from human umbilical vein: a) during incubation with histamine 10^{-5} M; b) with PMA 10^{-9} M; c) during exposure to hypoxia. Abscissa, time of action (in min); ordinate, total fraction of labeled inositol phosphates ($\cdot 10^3$ cpm/10^6 cells); d) ratio between basal and histamine-stimulated levels of PI metabolism (ordinate, per cent). Abscissa, time (in min). 1) Basal level, 2) histamine-stimulated level (10^{-5} M histamine, 5 min). ×) Incubation with histamine, ●) with PMA, △) during hypoxia.

er, and centrifuged for 10 min at 32,000g. The residue was resuspended in hypotonic buffer (protein concentration 1 mg/ml). Protein was determined by a modified method in [13]. When specific binding of the selective radioligand with membrane receptors was measured, ^{125}I-cyanopindolol ("Amersham," England) was used: the incubation time at 36°C was 60 min. Nonspecific binding was measured in the presence of 10^{-6} M D,L-propranolol, and specific binding was determined by subtracting nonspecific from total binding. Adenylate cyclase (AC) activity was determined in the membranes of EC with the aid of [^{32}P]-ATP [9]. To determine the intensity of metabolism, the preconfluent culture of EC was incubated for 48 h with myo-[^{3}H]-inositol (5 μCi/ml) in medium not containing endothelial growth factor. Before the experiment the cells were washed free from label with medium 199 and from serum and were incubated for 30 min in the presence of 10 mM LiCl. Incubation with 10^{-5} M histamine was carried out for 5 min at 37°C. The reaction was stopped by the addition of 1 ml of a solution containing 1% SDS and 30 mM EDTA. The lysate was applied to a column with 0.5 ml Dowex-4, 200-400 mesh (formate form), and inositol phosphate esters were eluted with a stepwise increasing concentration of ammonium formate [14]. To reproduce the conditions of hypobaric hypoxia, cells in open flasks or dishes were placed in a chamber with adjustable temperature and pressure (West Germany) and kept there for 1 h at a temperature of 37°C and a barometric pressure of 290 mm Hg. A statistical analysis of the data was carried out by Student's test for small samples.

EXPERIMENTAL RESULTS

A reduction of pO_2 by half in medium in which the EC were incubated took place 15 min after the atmospheric pressure was lowered to 290 mm Hg, with the result that increasing activation of hydrolysis of inositol phosphates (IP) was observed (Fig. 1c). Maximal accumulation of inositol phosphates, including IP_3, was found after exposure to hypobaric hypoxia for 40 min. Evidently when the oxygen supply of the cells was insufficient, increasing the intracellular calcium

TABLE 1. Number of β-Adrenergic Receptors (B_{max}) and AC-Activity of EC from Human Umbilical Vein During Exposure to Mediators and Hypoxia

Pre-incubation	B_{max}, fmoles/mg protein	AC, pmoles cAMP/min/mg protein	
		basal	+ isoproterenol
Control	14,6±3	33±1	52±4
Histamine (10^{-4} M)	4,6±2	12±6	22±2,5
PMA (10^{-10} M)	4,1±1,6	9±0,5	12±1,6
Hypoxia	9,8±1,3	18±2	22±3

concentration may take place not only on account of extracellular sources, but also as a result of its release from intracellular depots when the IP_3 level is raised. The problem of the mechanisms responsible for activation of phospholipase C during hypoxia, which catalyzes PI hydrolysis, remains unsolved. The participation of peroxide compounds and Ca^{2+} in this process can only be suggested [7].

The addition of histamine to intact EC led to an increase in their PI level and intracellular calcium concentration [5, 14]. A similar effect also was observed in our experiments (Fig. 1) and the addition of histamine to intact cells caused a two-threefold increase in the quantity of hydrolysis products of polyphophoinositides. However, activation of EC with 10^{-5} M histamine after hypoxia did not lead to stimulation of metabolism against the background of a raised basal level, or in other words hypoxia was the cause of the reduced sensitivity of Ca-mobilizing receptors to the agonists.

The results of the study of AC activity and of binding of the labeled ligand with β-receptors of EC after hypoxia are shown in Table 1. Clearly keeping EC under conditions with oxygen insufficiency led to depression of the basal and disappearance of the isoproterenol-stimulated AC activity, but with no significant changes in the number of β-receptors of EC. Investigations [11] showed that more prolonged (2 h) and deeper hypoxia (pO_2 < 1.5 To) led not only to weakening of the response of AC to the agonist, but also to a decrease in the number of β-receptors on the surface of cultured cardiomyocytes.

To study the possible mechanisms of desensitization of EC under hypoxic conditions, the dynamics of the change in PI metabolism, AC activity, and the number of β receptors during long-term (1-2 h) incubation of the cells with histamine (10^{-5} M), with phorbol-13-myristate-13-acetate (PMA) (10^{-9} M), a DAG analog which irreversibly activates PKC, was analyzed under hypoxic conditions. It was shown that EC lose their sensitivity to the activating action of histamine on PI metabolism as early as after 15 min of incubation with this Ca-mobilizing agonist (Fig. 1a). A similar dynamics was demonstrated also during incubation for 1 h with PMA (Fig. 1b). Thus direct activation of PKC reproduces the effects of long-term stimulation of the cells by histamine, evidence of the existence of negative feedback during long-term activation of the Ca-mobilizing receptors. Similar data obtained on EC from the human umbilical vein were given elsewhere [5].

Besides the "blocking" of the PI pathway of regulation during long-term incubation of EC with histamine or PMA, the number of β-adrenoreceptors and AC activity in the membrane of EC were considerably reduced (Table 1). Consequently, during the prolonged action of Ca-mobilizing agonists not only homologous, but also heterologous desensitization of the cAMP-dependent signal-conducting system of EC develops due both to a decrease in the number of β-receptors and blocking of hormonal sensitivity of AC to isoproterenol. Long-term activation of PKC followed by phosphorylation of proteins, which may include receptors and GTP-binding proteins, evidently leads to a change in the properties of the receptors and to their possible internalization [5, 10].

It has been suggested that elevation of the cytoplasmic ionized Ca^{2+} level during activation of PI metabolism is a short-term process, rapidly realized, for the number of calcium depots on which IP_3 acts is limited. The level of the second active metabolite of polyphosphoinoside hydrolysis, mainly DAG, remains elevated for a longer time, converting PKC into the active state and realizing the prolonged effect of the Ca-mobilizing hormones [12]. Interaction of the two intracellular messenger systems (AC and PI metabolism) takes place at different levels of the hormonal stimulus transmission cascade. The results of our investigations indicate that during the long term action of the hormone this interaction may be negative and may take place through activation of PKC, as is shown by the development of desensitization of Ca-mobilizing receptors and AC during incubation of EC with the DAG analog.

Comparison of the effects of hypoxia and Ca-mobilizing hormones on the signal-conducting systems of human EC suggests that oxygen insufficiency can simulate the effect of Ca-mobilizing hormones on PI metabolism and can suppress AC of the EC, modifying the sensitivity of the endothelium to vasoactive agents. The effects of hypoxia develop more slowly under these circumstances than the effects of Ca-mobilizing hormones.

LITERATURE CITED

1. V. A. Tkachuk, Introduction to Molecular Endocrinology [in Russian], Moscow (1983).
2. A. A. Abdel-Latif, Pharmacol. Rev., **38**, 227 (1986).
3. H. Acker, Ann. Rev. Physiol., **51**, 835 (1989).
4. M. J. Berridge, J. Biol. Chem., **367**, 447 (1986).
5. T. A. Brock and E. A. Capasso, J. Cell Physiol., **136**, No. 1, 54 (1988).
6. R. Busse, U. Pohl, and C. Kellner, Pflügers. Arch., **397**, No. 1, 78 (1983).
7. R. Busse, U. Forstermann, H. Matsuda, et al., Pflügers. Arch., **401**, No. 1, 77 (1984).
8. J. G. De Mey and R. M. Vanhoutte, Arch. Int. Pharmacodyn., **253**, 325 (1981).
9. G. Y. Grigorian (G. Yu. Grigoryan), T. Yu. Mirzapoyazova (T. Y. Mirzapoyazova), T. J. Resink, et al., J. Molec. Cell. Cardiol., **21**, Suppl. 1, 119 (1989).
10. K. H. Jacobs, Mol. Mech. Desensitiz. Signal Mol., Berlin (1987), pp. 83-93.
11. J. D. March and K. A. Sweeney, Am. J. Physiol., **256**, 257 (1989).
12. M. J. Mooibroek and J. H. Wang, Biochem. Cell Biol., **66**, No. 6, 557 (1988).
13. G. L. Peterson, Analyt. Biochem., **83**, 346 (1977).
14. T. J. Resink, G. Yu. Grigoryan (G. Y. Grigorian), A. K. Moldabaeva, et al., Biochem. Biophys. Res. Commun., **144**, 438 (1987).