Dynamics of Intracellular Superoxide and NO Content in Human Endotheliocytes and Carcinoma Cells after Treatment with NO Synthase Inhibitors

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The dynamics of intracellular levels of superoxide and NO after cell treatment with NO synthase inhibitors were studied in human cells expressing various NOS isoforms: endotheliocytes and ECV-304 (eNOS) and carcinoma cells and HeLa-G63 (iNOS). Cytometric analysis of changes in the cell fluorescence intensity was carried out using superoxide and NO fluorescent indicators (dihydroethidene and DAF-2-DA, respectively). Intracellular levels of superoxide decreased in HeLa-G63 and ECV-304 cells after their incubation in medium with aminoguanidine, L-NAME, and D-NAME. Intracellular NO level decreased only in HeLa-G63 cells after incubation in medium with aminoguanidine and L-NAME, but not D-NAME. The level of NO returned to normal after 7-h culturing in inhibitor-free medium, while the level of superoxide increased and remained high throughout 3 generations. Incubation of cells with D-NAME did not increase the intracellular level of superoxide. Presumably, high prolonged generation of superoxide is a delayed result of inhibition of NO synthesis in HeLa-G63 cells.

Key Words: *superoxide; NO synthase inhibitors; cytometry; HeLa; ECV-304*

The NO is produced in cells from L-arginine by oxidation of guanidine fragment amino group nitrogen with participation of constitutive and inducible NO synthase isoforms. All NO synthase isoforms utilize L-arginine, oxygen, and NADPH as the substrates for NO synthesis. Tetrahydrobiopterin (BH_4, calmodulin, flavinadenine dinucleotide (FAD), and flavin mononucleotide (FMN) are obligatory co-factors of this catalytical process [9]. Inducible (iNOS) and neural (nNOS) NO synthases produce superoxide under conditions of reduced level of L-arginine, while endothelial NO synthase (eNOS) produces superoxide when BH_4 level is reduced [6,13]. The fact that NO synthase inhibitors reduce the content of reactive oxygen species (ROS) suggests that NO synthase could be a source of ROS [8]. Endogenously produced NO acts as a signal and effector molecule in the cardiovascular, neural, and immune systems. This wide spectrum of NO and superoxide activities necessitates constant search for approaches to regulation of these radicals in the cell. An important factor in this search is the method for their registration in the cells under normal conditions.

We carried out a life-time evaluation of the dynamics of intracellular superoxide and NO in human endotheliocytes and carcinoma cells after their treatment with NO synthase inhibitors.

MATERIALS AND METHODS

Intracellular superoxide level was evaluated using dihydroethidine (Sigma), a fluorescent indicator of superoxide anion. Superoxide interacts with dihydroethidine yielding oxyethidium (Etd^+), a specific fluorescent product. The level of NO in cells was evaluated using 4,5-diaminofluorescein diacetate (DAF-2-DA; Cay-

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man) transformed into DAF-2 in the cell. DAF-2 molecules interact with NO in a concentration-dependent mode and produce highly fluorescent triazolfluorescein (DAF-2-T) [10]. Cytometric evaluation of NO and superoxide levels in cells is based on registration of DAF-2-T green fluorescence (510 nm) and Etd^+ red fluorescence (610 nm). Cell suspension was analyzed on a flow cytometer created at our Institute [5]. The fluorescence was recorded in a semilogarithmic ($\log_2$) scale, at which the 40 channel difference between the fluorescence peaks corresponded to a change in intracellular level of the measured radical by 2 times. At least 10,000 cells were analyzed for each sample. Each experiment was repeated at least 4 times.

The study was carried out on two cell strains expressing various NOS isoforms: ECV-304 human endotheliocytes (eNOS) and HeLa-G63 human carcinoma cells (iNOS). We previously showed that these cell strains differ by intracellular NO level and modification of this level by NO synthase inhibitors [1,2]. Both cell strains were cultured in modified Eagle's medium (ICN) with 10% fetal serum (Biolot) and gentamicin.

Aminoguanidine (AG; Ferak), L-NAME (ω-N-nitro-L-arginine methyl ester; Sigma) served as NO synthase inhibitors. Aminoguanidine is a specific inhibitor of iNOS, L-NAME inhibits all three NOS isoforms. D-NAME (ω-N-nitro-D-arginine methyl ester), inactive isomer of L-NAME, served as an additional control.

RESULTS

Both cell strains were cultured in Carrel's flasks in media with NO synthase inhibitors AG, L-NAME, and D-NAME in a final concentration of 0.15 mM and in inhibitor-free medium. The shortest incubation of cells with inhibitors was 4 h, after which the medium was discarded, the cells were removed with versen, a portion of cell suspension was stained with dihydroethidine and the other portion was cultured

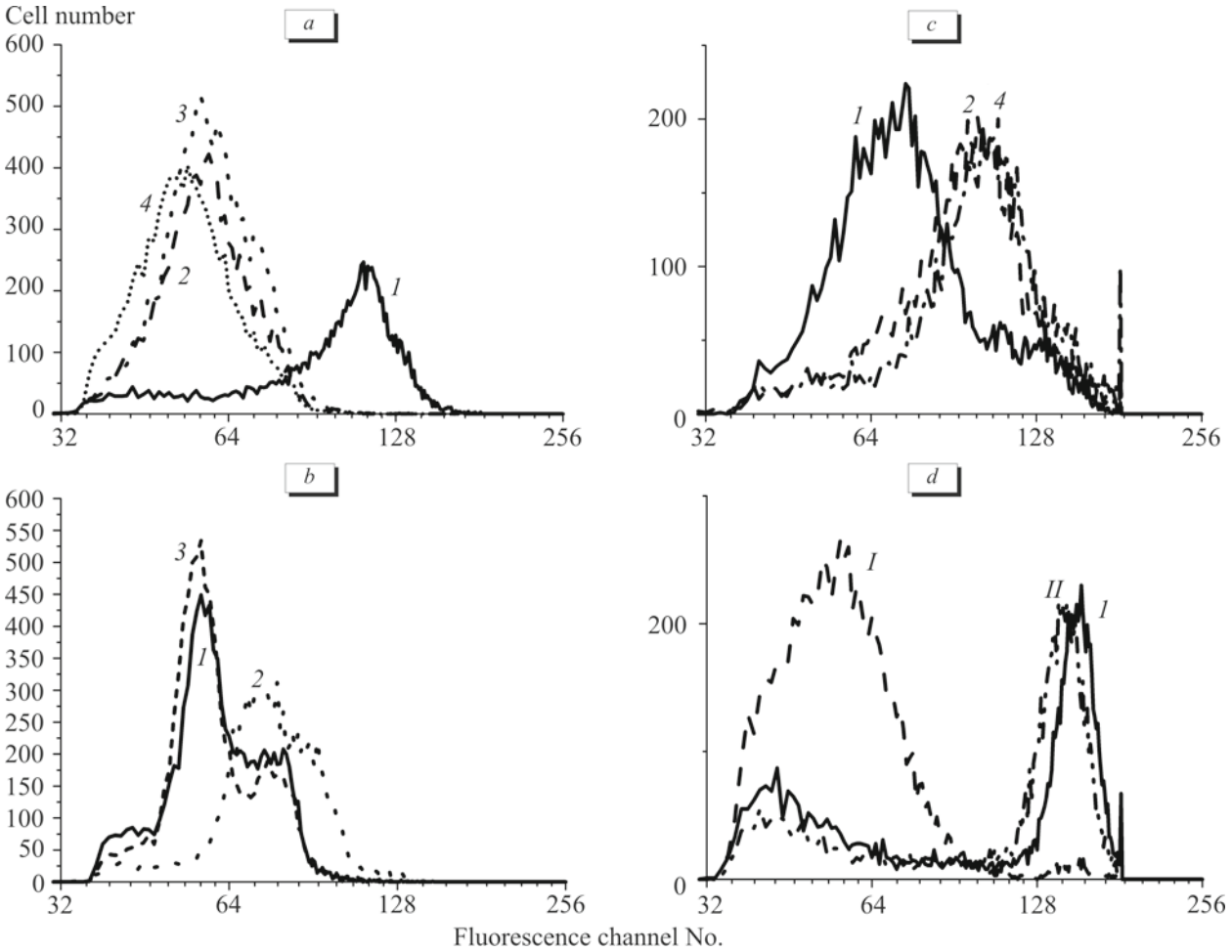

Fig. 1. Effects of NO synthase inhibitors on intracellular levels of superoxide and NO in HeLa cells. *a*) intensity of fluorescence of cells stained with dihydroethidine directly after removal of inhibitors from nutrient medium; *b*) 7 h after removal; *c*) 24 h after removal; *d*) intensity of fluorescence of cells stained with DAF-2-DA directly (I) and 7 h (II) after removal of L-NAME from nutrient medium. Here and in Fig. 2: *1*) no inhibitors; after 4-h treatment with L-NAME (*2*), D-NAME (*3*), and AG (*4*).

in fresh nutrient medium for 24-72 h more. All the studied reagents reduced HeLa cell fluorescence intensity by 56 channels in comparison with intact control, which corresponded to 2.8-fold reduction of intracellular level of superoxide (Fig. 1, *a*). The absence of appreciable differences in the efficiency of L-NAME and D-NAME suggests that the decrease in superoxide concentration observed directly after 4-h treatment was most likely caused by scavenger activity of these agents, which agrees with the results of other studies [11]. After 7-h culturing in free medium, the intensity of fluorescence of cells treated with L-NAME and D-NAME increased. Fluorescence intensity in cells treated with L-NAME even surpassed the control, while for cells treated with D-NAME this parameter did not surpass the level characteristic of the control (Fig. 1, *b*). It seems that scavenger effect disappears with time and the difference between the efficiencies of these two isomers at this stage of cell fixation is determined by inhibition of NO synthase activity. Analysis of fluorescence intensity in cells treated with AG and L-NAME for 4 h and then cultured for 24 h in inhibitor-free medium revealed an increase of intracellular superoxide level (by 1.7 times; a shift of the fluorescence peak by 35 channels in comparison with the control; Fig. 1, *c*). Elevated intracellular superoxide level was recorded in the cells throughout three generations. We hypothesized that prolonged effect of high generation of superoxide was caused by lasting reduction of intracellular NO level under the effect of the inhibitors. However, analysis of changes in intracellular NO in HeLa cells directly and 7 h after their incubation with L-NAME (Fig. 1, *d*) showed that 7 h after removal of the inhibitor from the medium the intracellular NO level reduced significantly after L-NAME treatment returned to the control level. The absence of D-NAME effect on delayed generation of the superoxide and similar effects of two NO synthase inhibitors with different mechanisms of action (L-NAME inhibits NO synthase activity at the expense of competitive binding, while AG inhibits glycation process) indicate that it is the inhibition of NO synthase synthesis that leads to hyperproduction of the superoxide by the cells. We obtained similar data on other human tumor cell strain, T-24 (bladder epithelium); the data are not presented. The results are in line with the results of studies on other cell systems. An *in vivo* study on rats showed that prolonged exposure to NO synthase inhibitor stimulated free radical oxidation in cardiomyocytes and lung epithelial cells [3]. It was also shown that long-term inhibition of NO synthase activity leads to oxidative injuries through an increase of xanthine oxidase activity [4,8]. The assumption that long-term effects of L-NAME are mediated mainly through inhibition of NO synthase remains disputable. Numerous nonspecific effects of L-NAME were recorded [12]. Incubation of ECV-304 cells with L-NAME and D-NAME led to reduction of cell fluorescence intensity by just 10 channels after dihydroethidine treatment and did not lead to fluorescence intensity reduction after cell treatment with DAF-2-DA (Fig. 2, *b*). These results confirmed our previous data [2] and the results of other authors demonstrating that the effect of L-NAME on the vascular systems is not determined by inhibition of endothelial NO-synthase [12]. The patterns of NO and ROS synthesis regulation largely coincide: both are activated by the same antigens, have the same intracellular targets, and act in parallel. Simultaneous evaluation of changes in these radicals in the cell under the effects of drugs widely used in medicine (for example, AG and L-NAME) will promote better understanding of the processes leading

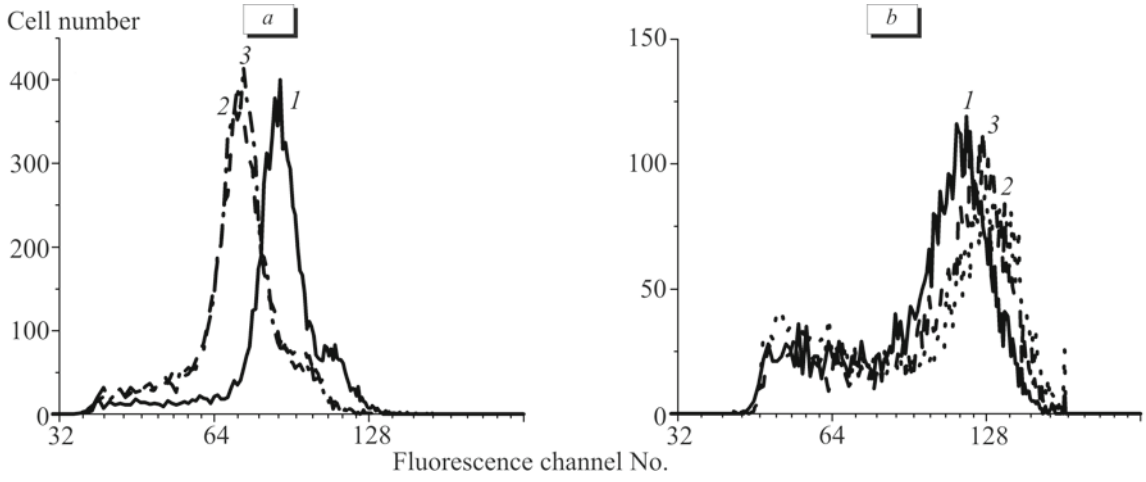

Fig. 2. Effects of L-NAME and D-NAME on intracellular levels of superoxide and NO in ECV-304 cells. *a*) intensity of fluorescence of cells stained with dihydroethidine directly after removal of inhibitors from nutrient medium; *b*) intensity of fluorescence of cells stained with DAF-2-DA directly after removal of inhibitors from nutrient medium.

to oxidative stress and evaluation of the possibility of drug correction of this effect, because enhanced long-term generation of superoxide can trigger many pathological changes in the cells, from modification of DNA bases to carcinogenesis.

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