Intracellular NO Concentration and Its Changes in Carcinoma Cells and Cultured Human Endotheliocytes under the Influence of Inhibitors and Inductors of Nitric Oxide Synthases

N. Ya. Gil'yano, S. I. Stepanov, L. V. Konevega, and L. A. Noskin

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 143, No. 2, pp. 211-213, February, 2007
Original article submitted May 15, 2006

We carried out cytophotometric evaluation of NO content in cells expressing various types of NO synthases. Endogenous production of NO can be modified by NO synthase inductors and inhibitors.

Key Words: *nitric oxide; 4,5-diaminofluorescein diacetate; HeLa; ECV 304*

NO is a radical produced by various cells from L-arginine in complex oxidative reactions catalyzed by NO synthases (NOS). The role of free radicals in the regulation of signal transduction and other biological functions remains poorly studied due to difficulties in quantitative evaluation of these agents under physiological conditions [9]. Online monitoring of NO concentration in living cells is difficult, because it is a short-living (6-10 sec) and highly reactive compound, which reacts with O_2 molecule, superoxide anion, and metals ions of alternating valence. NO concentration in living cells is measured with fluorescent indicators of NO, including 4,5-diaminofluorescein diacetate (DAF-2-DA) [6,7]. This indicator easily penetrates into cells, ester bonds of this substance are hydrolyzed by intracellular esterases with the formation of a relatively nonfluorescent membrane-impermeable DAF-2. Rapid, irreversible, and concentration-dependent interaction of DAF-2 with NO in the solution yields highly fluorescent triazolfluorescein (DAF-2-T). DAF-2-T is formed only in the presence of NO. Stable nitrogen oxides, including NO_2^- and NO_3^-, reactive oxygen species O_2, H_2O_2, and $ONOO^-$ do not react with DAF-2 [7]. The specificity and high sensitivity of NO-reactive fluorescent indicators allow us to perform online motoring of intracellular NO concentration.

This work was designed to perform a cytophotometric study of intracellular NO using DAF-2-DA. The type of NOS responsible for NO production was estimated. We evaluated whether endogenous NO production can be regulated by NOS inductors and inhibitors.

MATERIALS AND METHODS

Experiments were performed on human ECV 304 endotheliocytes and carcinoma HeLa cells expressing endothelial (eNOS) and inducible NOS (iNOS), respectively. The cells were cultured in modified Eagle medium (ICN) containing 10% fetal bovine serum (Biolot) and gentamicin. DAF-2-DA (ALEX) served as a fluorescent indicator of NO.

For NO induction we used ^{3}H-thymidine in low concentration (0.066 MBq/ml). Long-term treatment with ^{3}H-thymidine (irradiation on low doses) induced iNOS in various cell types [5,11].

Aminoguanidine (Ferak), N^{ω}-nitro-L-arginine methyl ester (L-NAME, Sigma), and inactive iso-

B. P. Konstantinov Petersburg Institute of Nuclear Physics, Russian Academy of Sciences, Gatchina. ***Address for correspondence:*** giliano@omrb.pnpi.spb.ru. N. Ya. Gil'yano

0007-4888/07/1432-0248

mer N^{ω}-nitro-D-arginine methyl ester (D-NAME) served as NOS inhibitors. Aminoguanidine is a specific iNOS inhibitor, which inhibits all 3 types of NOS. D-NAME was used as an additional control.

The cell suspension was studied on a cytofluorometer constructed at the Department of Molecular and Radiation Biophysics (B. P. Konstantinov Petersburg Institute of Nuclear Physics) [10].

For cytometry, the cells were incubated in Carrel flasks in the presence or absence of ^{3}H-thymidine and were examined after 48 h. Aminoguanidine, L-NAME, and D-NAME were added to the nutrient medium 2 h before fixation. The cell monolayer was harvested with Versene. The cell suspension was incubated in the presence or absence of DAF-2-DA (0.01-1.00 μg/ml) at 37°C. The time of incubation varied from 15 min to 1 h. Nine independent experiments were performed to develop the conditions optimal for measurement of green fluorescence (selection of filters and minimization of the effect of scattering) and vital study of endogenous NO in cells (concentration of a staining agent, time of incubation, conditions of incubation, and time of recording). Fluorescence was recorded in the half-logarithmic scale. The difference of 40, 30, 20, and 10 channels corresponded to variations in NO concentration by 2, 1.6, 1.41, and 1.19 times, respectively.

RESULTS

The peaks of green fluorescence (510 nm) were determined by the formation of DAF-2-T after 15-min incubation of cells with 0.5 μg/ml DAF-2-DA (Fig. 1, *a*). Fluorescence of HeLa cells was 1.4 times higher compared to that of ECV 304 cells, which confirmed the hypothesis that HeLa cells express iNOS. Fluorescence peak observed in ECV 304 cells confirmed high sensitivity of the method, which allowed us to detect low concentration of NO produced by eNOS. ECV 304 cells incubated in the absence of the dye did not fluoresce in this range of the spectrum, which excluded the contribution of scattering into the fluorescence peak. Fluorescence significantly increased after 30-min incubation of HeLa cells in a medium with ^{3}H-thymidine (0.066 MBq/ml). Four independent experiments showed that the fluorescence peak is shifted by 30 channels. The data showed that fluorescence and, therefore, intracellular NO concentration increased by 1.5 times. It can be hypothesized that low doses of β-radiation induced NO production in cells. Fluorescence decreased by 60 channels after 2-h pretreatment of HeLa cells with 0.07 mM L-NAME. These changes correspond to 3-fold decrease in intracellular NO concentration. A selective iNOS inhibitor aminoguanidine and L-NAME decreased

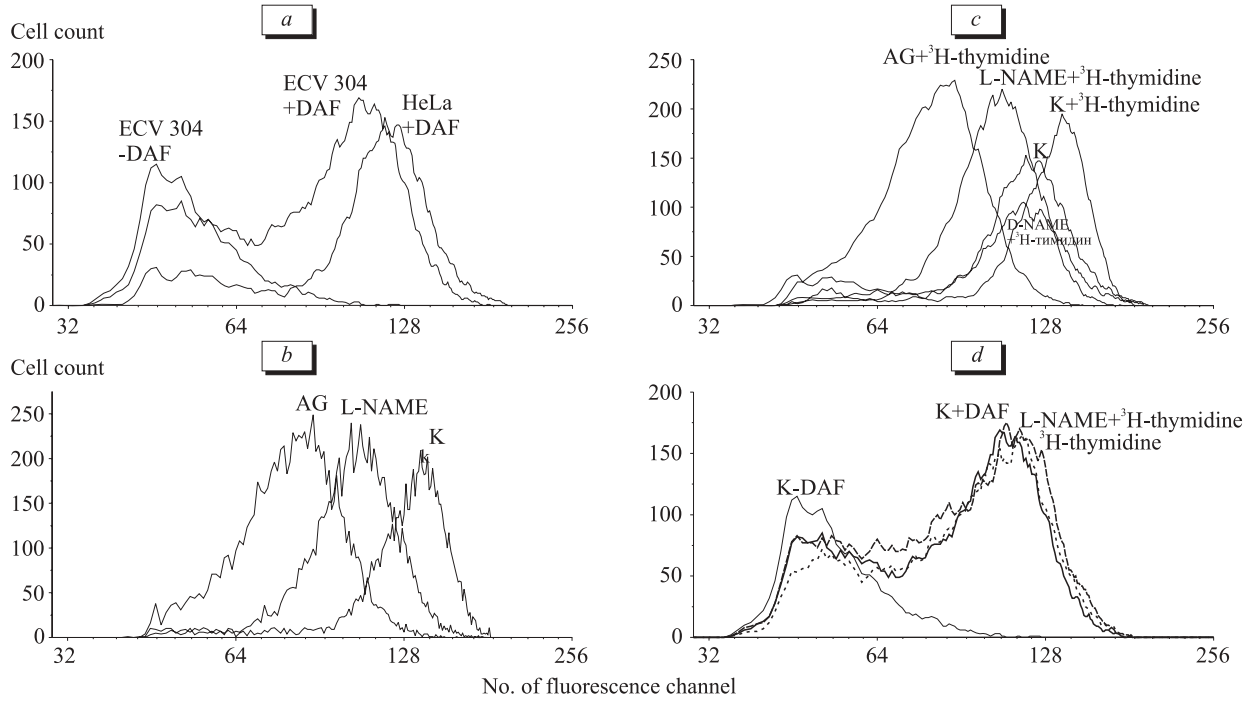

Fig. 1. Fluorescence of DAF-2-T in HeLa and ECV 304 cells. Basal NO level in cells after incubation in the presence or absence of DAF-2-DA (*a*); decrease in NO concentration in HeLa cells after 60-min incubation in the medium with L-NAME or aminoguanidine (*b*); increase in NO concentration after incubation of HeLa cells with ^{3}H-thymidine and decrease in NO concentration after 60-min incubation of cells in the medium containing L-NAME, aminoguanidine, and D-NAME (*c*); no changes in NO concentration in ECV 304 cells after 60-min incubation in the medium with L-NAME, D-NAME, and ^{3}H-thymidine (*d*).

the intensity of fluorescence. The fluorescence peak was shifted by only 30 channels after pretreatment of cells with 0.07 mM D-NAME (2-fold lower compared to the effect of L-NAME). The decrease in fluorescence intensity after cell treatment with inactive isomer of NOS inhibitor suggests that the decrease in intracellular NO concentration is realized via both enzymatic (inhibition of NOS activity) and nonenzymatic pathways (radical trapping). L-NAME and D-NAME serve as free radical-trapping agents. Since L-NAME inhibits NOS, this compound is more potent in regulating intracellular NO concentration [8].

Pretreatment of ECV 304 cells with L-NAME, D-NAME, or aminoguanidine in a concentration of 0.07 mM had little effect on fluorescence (Fig. 1, *d*). Increasing the concentration of L-NAME to 3 mM was accompanied by a decrease in fluorescence of ECV 304 cells. However, these changes were less pronounced compared to HeLA cells. Published data show that L-NAME in millimolar concentrations primarily acts as a radical-trapping agent [8]. Our previous studies showed that L-NAME produces a radioprotective effect. It was estimated from the incidence of chromosome aberrations in human carcinoma HeLa cells and Chinese hamster V-79 fibroblasts irradiated with various doses of ^{137}Cs γ-quanta, ^{14}C β-particles, and fission neutrons. The absence of radioprotective activity of D-NAME suggests the existence of a NO-dependent mechanism for modification of cell radiosensitivity [1,3]. Radioprotective properties were also typical of selective iNOS inhibitor aminoguanidine. NOS inhibitors produced no radioprotective effect in human ECV 304 endotheliocytes [2]. These data confirm our hypothesis, since endotheliocytes belong to NO-resistant cells [4]. A direct correlation probably exists between variations in intracellular NO concentration and modifiability of the radiation effect in cell genetic structures. Different effectiveness of endogenous NOS inhibitors is probably associated with activity of the system for regulation of high-reactivity NO derivatives in these cell lines.

We are grateful to E. G. Bikineeva and A. L. Konevega for their help in this study.

This work was supported by the Russian Foundation for Basic Research (grant No. 05-04-49261-a).

REFERENCES

1. N. Ya. Gil'yano, G. N. Bondarev, E. G. Bikineeva, *et al*., *Radiats. Biol. Radioekol.*, **41**, No. 6, 653-658 (2001).
2. N. Ya. Gil'yano, G. N. Bondarev, L. V. Konevega, *et al*., *Ibid*., **45**, No. 1, 63-67 (2005).
3. N. Ya. Gil'yano, G. N. Bondarev, L. A. Noskin, *et al*., *Ibid*., **44**, No. 1, 62-67 (2004).
4. S. Dimmeler, J. Haendeler, M. Nehls, and A. M. Zeiher, *Exp. Med*., **185**, No. 4, 601-607 (1997).
5. K. Ikeya, S. Kashimoto, M. Kume, and T. Kumazawa, *Masui*, **50**, No. 4, 365-370 (2001).
6. H. Kojima, N. Nakatsubo, K. Kikuchi, *et al*., *Anal. Chem*., **70**, No. 13, 2446-2453 (1998).
7. T. Nagano, *Luminescence*, **14**, No. 6, 283-290 (1999).
8. A. Rehman, M. Whiteman, and B. Halliwell, *Br. J. Pharmacol*., **122**, No. 8, 1702-1706 (1997).
9. J. S. Stamler, D. J. Singel, and J. Loscalzo, *Science*, **258**, No. 5090, 1898-1902 (1992).
10. S. I. Stepanov, V. N. Konyshev, L. V. Kotlovanova, *et al*., *Cytometry*, **23**, No. 4, 279-283 (1996).
11. N. V. Voevodskaya and A. F. Vanin, *Biochim. Biophys. Res. Commun*., **186**, No. 3, 1423-1428 (1992).