Effects of Endothelin-1 on DNA Synthesis and NADPH Diaphorase Activity in Epithelial and Smooth Muscle Cells of the Tracheobronchial System in Newborn Albino Rats after Repeated Treatment with N^G-Nitro-L-Arginine Methyl Ester

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Newborn rats received intraperitoneal injections of endothelin-1 in a daily dose of 5×10^{-8} mol/kg 30 min after administration of N^G-nitro-L-arginine methyl ester (9.3×10^{-5} mol/kg intraperitoneally) from the 2nd to 6th day of life. NADPH diaphorase activity in epithelial and smooth muscle cells of the tracheobronchial system increased 24 h after the last treatment. In this period DNA synthesis underwent opposite changes, which included the inhibition of epithelial cell proliferation and stimulation of smooth muscle cell division. Intensification of free radical oxidation in the lung tissue was accompanied by inactivation of the antioxidant system.

Key Words: *endothelin; nitric oxide; DNA synthesis; epithelial cells; smooth muscle cells; respiratory tract*

Nitric oxide (NO) synthesized by NO synthases (NOS) plays a key role in the formation of bronchomotor tone in developing lungs and counteracts the adverse effects of exo- and endogenous constrictors, including endothelin-1 (ET-1). An imbalance in the expression of bronchoactive agents during the early ontogeny can induce pathological changes in the growth, formation, and activity of the tracheobronchial system. Here we studied the effects of ET-1 on DNA synthesis, NADPH diaphorase activity in epithelial and smooth muscle cells of the trachea and cartilaginous and non-cartilaginous bronchi, and free radical oxidation in the lungs of newborn albino rats after repeated treatment with NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME).

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MATERIALS AND METHODS

Experiments were performed on 105 newborn albino rats. Control and experimental groups were composed by the method of litter separation to reduce genetically determined differences between litters. The animals received intraperitoneal injections of test substances at 10.00-11.00 from the 2nd to 6th day of life. Group 1 and 2 rats were injected with 5×10^{-8} mol/kg ET-1 and 9.3×10^{-5} mol/kg L-NAME, respectively. Group 3 rats received ET-1 30 min after administration of L-NAME. Control animals were injected with isotonic NaCl. The rats were decapitated 24 h after the last treatment. DNA synthesis was studied by autoradiography. The rats were intraperitoneally injected with ^{3}H-thymidine in a dose of 1 mCi/g (specific activity 1570 TBq/mol)

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1 h before decapitation. Autoradiographs were prepared routinely. The number of S-phase cells (index of labeled nuclei, ILN, %) and mean number of silver grains over the nucleus (labeling intensity, LI) were counted in epithelial and smooth muscle cells of the trachea and cartilaginous and noncartilaginous bronchi. Histochemical assay for NADPH diaphorase (NOS marker, Fig. 1) was performed by the method described elsewhere using a single-beam photometer equipped with an FMEL-1Uch.2 attachment at 550 nm [9]. The method of H_2O_2-induced luminol-dependent chemiluminescence was used to estimate the intensity of free radical oxidation [1]. Chemiluminescence was recorded on an LS 50B luminescence spectrometer (Perkin Elmer). Signals were standardized using Finlab software. The intensity of spontaneous (over 1 min) and H_2O_2-induced luminol-dependent chemiluminescence (over 2 min) and maximum flash amplitude (I_{max}) were measured at room temperature, calculated per 1 mg lipids, and expressed in arbitrary units. Total lipid content was estimated by the phosphovanillin method using Lachema kits.

The results were analyzed by Student's *t* test.

RESULTS

Administration of ET-1 significantly decreased ILN and LI in tracheal epithelial cells (by 1.5 and 1.3 times, respectively, compared to the control). Moreover, ILN in cartilaginous and noncartilaginous bronchi decreased by 1.4 times (Table 1). ET-1 increased ILN and LI in tracheal smooth muscle cells by 1.6 and 1.2 times, respectively. In smooth muscle cells of cartilaginous and noncartilaginous bronchi ET-1 increased only ILN (by 1.6 and 1.5 times, respectively, Table 1). NADPH diaphorase activity in epithelial cells of the trachea and cartilaginous and noncartilaginous bronchi increased by 1.2, 1.3, and 1.3 times, respectively (Table 1). The intensity of H_2O_2-induced chemiluminescence and I_{max} in lung homogenates increased by 1.5 times (Table 2).

Therefore, ET-1 inhibited DNA synthesis and activated NOS in epithelial cells, stimulated proliferation of smooth muscle cells in the respiratory tract, and intensified free radical oxidation in the lung tissue. These results suggest that morphological changes induced by ET-1 are realized via the NO—NOS system and free radical oxidation. ET-1 interacts with ET_A receptors in the epithelium, activates NO synthesis in epithelial cells and, therefore, counteracts peptide-induced bronchoconstriction [12]. It should be emphasized that ET-1 possessing antiinflammatory activity can activate epithelial NOS by some other mechanisms. Our previous experiments and published data show that ET-1 induces overproduction of free radicals, in particular superoxide anion radicals [5], in the lungs [11]. This is accompanied by activation of nuclear factor NF-κB [2] involved in iNOS gene transcription [15]. NO excess can inhibit constitutive NOS, in particular eNOS [14]. Inhibition of DNA synthesis in the epithelium after repeated treatment with ET-1 is probably related to oxidative tissue damages. The reaction between superoxide anions and NO results in the formation of peroxynitrite, which is highly toxic for the epithelium [3]. The nature of asthmatic damages to the respiratory epithelium is similar. Immunohistochemical assay confirmed the formation of peroxynitrite in the respiratory tract and, particularly, in the epithelium [6]. The mitogenic effect of ET-1 on smooth muscle cells in the respiratory tract can be realized not only via the direct interaction of ET-1 with ET_A receptors [13], but also via the epithelium-

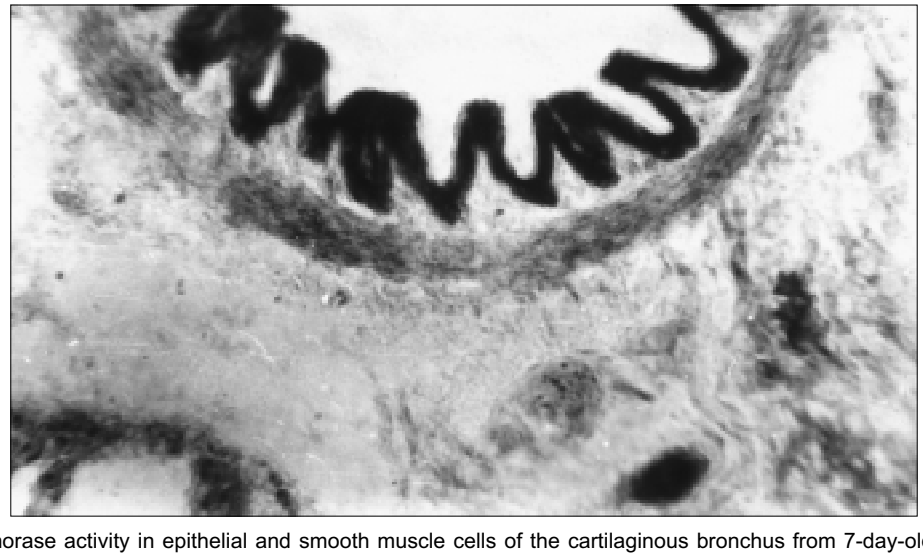

Fig. 1. NADPH diaphorase activity in epithelial and smooth muscle cells of the cartilaginous bronchus from 7-day-old rat (control). Hope—Vincent staining (×75).

NO mechanism. Under physiological conditions NO constitutively synthesized in the respiratory tract produces a protective antiproliferative effect on smooth muscle cells [7]. Overproduction of NO and desensitization of soluble guanylate cyclase abolish the protective effects of NO [8].

The effects of L-NAME on the respiratory tract in newborn rats were similar to those induced by ET-1. L-NAME decreased ILN in epithelial cells of the trachea and cartilaginous and noncartilaginous bronchi by 1.4, 1.3, and 1.4 times, respectively. Treatment with L-NAME significantly increased ILN in smooth muscle cells of the trachea and cartilaginous bronchi (by 1.5 and 1.6 times, respectively). ILN and LI in noncartilaginous bronchi increased 1.1- and 1.2-fold, respectively (Table 1). NADPH diaphorase activity increased in epithelial and smooth muscle cells of the trachea (by 1.6 and 1.2 times, respectively) and cartilaginous (by 1.3 and 1.5 times, respectively) and noncartilaginous bronchi (by 1.4 and 1.6 times, respectively, Table 1). The intensity of H_2O_2-induced chemiluminescence and I_{max} in the lungs increased by 1.6 and 1.7 times, respectively (Table 2).

Our experiments show that L-NAME inhibited DNA synthesis in epithelial cells, stimulated this process in smooth muscle cells, activated NOS in both cell types (as differentiated from ET-1), and induced accumulation of free radicals in the lungs. The effects of L-NAME inhibiting constitutive NOS [4] are probably realized via the free radical mechanism and NO—NOS system (similarly to ET-1). Repeated treatment with L-NAME induced expression of proteins possessing prooxidant and proinflammatory properties in vascular endotheliocytes and smooth muscle cells. Immunohistochemical assay and reaction for NADPH diaphorase demonstrated that L-NAME induced overproduction of iNOS in smooth muscle cells [10].

Pretreatment with L-NAME practically did not modulate proliferative activity of ET-1. L-NAME only slightly potentiated the proliferative effect of ET-1 on epithelial cells in noncartilaginous bronchi. Under these conditions ILN and LI decreased in epitheliocytes

TABLE 1. Effects of ET-1 and L-NAME on DNA Synthesis and NADPH Diaphorase Activity (Optical Density Units) in Epithelial and Smooth Muscle Cells of the Tracheobronchial System in Newborn Albino Rats (*M*±*m*)

Series	Epithelial cells			Smooth muscle cells		
	ILN	LI	NADPH diaphorase	ILN	LI	NADPH diaphorase
Control						
trachea	1.99±0.13	20.12±1.22	0.492±0.016	0.537±0.041	21.19±1.15	0.217±0.009
cartilaginous bronchi	1.86±0.12	21.33±1.25	0.438±0.012	0.526±0.034	20.82±1.18	0.178±0.008
noncartilaginous bronchi	1.67±0.10	22.12±1.14	0.39±0.01	0.520±0.038	19.51±1.08	0.150±0.008
ET-1						
trachea	1.32±0.09*	16.00±1.17*	0.59±0.02*	0.869±0.065*	25.85±1.39*	0.226±0.010
cartilaginous bronchi	1.30±0.08*	16.15±1.09*	0.584±0.021*	0.854±0.071*	25.4±1.2*	0.170±0.008
noncartilaginous bronchi	1.17±0.08*	19.12±1.28	0.531±0.017*	0.780±0.059*	23.78±1.17*	0.168±0.009
L-NAME						
trachea	1.44±0.10*	23.24±1.29	0.558±0.019*	0.788±0.065*	21.41±1.36	0.260±0.011*
cartilaginous bronchi	1.45±0.11*	20.00±1.21	0.528±0.020*	0.854±0.071*	20.65±1.12	0.253±0.015*
noncartilaginous bronchi	1.22±0.10*	23.33±1.20	0.453±0.018*	0.775±0.062*	19.87±1.22	0.234±0.015*
L-NAME+ET-1						
trachea	1.48±0.08*	15.25±1.13*	0.683±0.022*+o	0.920±0.083*	25.28±1.21*	0.384±0.016*+o
cartilaginous bronchi	1.35±0.11*	14.23±1.12*	0.667±0.020*+o	0.892±0.080*	25.73±1.53*	0.347±0.015*+o
noncartilaginous bronchi	1.1±0.1*	14.12±1.08*	0.634±0.020*+o	0.834±0.076*	25.12±1.32*	0.305±0.016*+o

Note. Here and in Table 2: $p<0.05$: *compared to the control, +compared to ET-1, °compared to L-NAME.

TABLE 2. Effects of ET-1 and L-NAME on H_2O_2-Induced Luminol-Dependent Chemiluminescence in Lung Homogenates from Newborn Albino Rats (*M*±*m*)

Parameter	Control	ET-1	L-NAME	L-NAME+ET-1
Intensity of chemiluminescence				
spontaneous	19.30±1.68	21.83±1.90	21.62±2.15	32.41±3.18*
H_2O_2-induced	57.72±4.22	84.75±7.12*	91.52±8.26*	116.34±9.48*+
I_{max}	0.470±0.035	0.720±0.053*	0.790±0.048*	1.140±0.102*+o

of the trachea (by 1.8 and 1.4 times, respectively) and cartilaginous (by 1.7 and 1.4 times, respectively) and noncartilaginous bronchi (by 1.9 and 1.6 times, respectively). Moreover, ILN and LI increased in smooth muscle cells of the trachea (by 1.7 and 1.3 times, respectively) and cartilaginous (by 1.7 and 1.2 times, respectively) and noncartilaginous bronchi (by 1.6 and 1.3 times, respectively, Table 1). NADPH diaphorase activity considerably increased (compared to the control) in epithelial and smooth muscle cells of the trachea (by 1.4 and 1.8 times, respectively) and cartilaginous (by 1.5 and 1.9 times, respectively) and noncartilaginous bronchi (by 1.6 and 2.0 times, respectively). Moreover, enzyme activity in rats injected with ET-1 and L-NAME surpassed that in animals receiving only ET-1 or L-NAME (Table 1). The intensity of spontaneous and H_2O_2-induced chemiluminescence and I_{max} in the lungs increased by 1.8, 2.0, and 2.4 times, respectively, compared to the control. It should be emphasized that the intensity of H_2O_2-induced chemiluminescence markedly surpassed that observed after treatment with ET-1. In these rats I_{max} was higher than in animals receiving only ET-1 or L-NAME (Table 2). Therefore, the proliferation-stimulating effect of ET-1 and damage to the NO-NOS system in epitheliocytes and smooth muscle cells of the respiratory tract are potentiated against the background of inhibition of constitutive NO synthesis, which can aggravate disturbances in the free radical balance in lung tissues.

Our findings suggest that ET-1 and NO-NOS system in epithelial and smooth muscle cells are involved in pathological remodeling of the respiratory tract in newborn rats.

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