Doxorubicin Modulates Biological Effects of ATP in Bone Marrow Cells *In Vitro*

Yu. A. Uspenskaya, A. B. Egorova*, and V. P. Nefedov

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ATP activity in mouse bone marrow cells was studied in *in vitro* experiments. ATP in physiological and supraphysiological concentrations stimulated blebbing of the plasma membrane. Subacute administration of doxorubicin (inductor of oxidative stress) decreased cell sensitivity to ATP, which corresponded to changes in membrane lipid peroxidation.

Key Words: *bone marrow; blebbing; ATP; doxorubicin*

Active metabolites modulating cell functions by the mechanisms of paracrine or autocrine regulation can be secreted and transported from cell or released into extracellular space after cytolysis. ATP interacting with purinergic membrane receptors possesses pronounced regulatory activity. Stimulation of purinergic receptors induces apoptosis of vascular endothelial cells, thymocytes, and some tumor cells [3,12]. The presence of $P7_{2Y}$ and P_{2X}/P_{2Z} receptors on bone marrow cells (BMC) was demonstrated [4]. ATP induces rapid rearrangement of the cytoskeleton and blebbing of the plasma membrane [7]. Blebbing is determined by local ionic (in particular, calcium) and energy imbalances in the perimembrane cytoplasm, structural rearrangement of the cytoskeleton, activation of signal cascade processes involving mitogen- and stress-activated protein kinases, myosin light chain kinases, and tissue transglutaminase [6,8].

Here we studied the effects of myelotoxic xenobiotic doxorubicin on ATP-induced blebbing in mouse BMC *in vivo* and *in vitro*.

MATERIALS AND METHODS

The study was carried out on albino outbred male mice weighing 20-25 g (5 animals per series).

Department of Anatomy and Physiology of Agricultural Animals, Krasnoyarsk State Agrarian University; *Department of Pathophysiology and Functional Diagnostics, Krasnoyarsk State Medical Academy. ***Address for correspondence:*** onmpi@krsk.infotel.ru. Egorova A. B.

Blebbing was studied by phase contrast microscopy. BCM were incubated in medium 199 (10^6/ml) at 37°C for 5, 15, 30, 45, and 60 min and 200 cells in each preparation were analyzed under immersion microscope (×900). Small (initial blebbing) and large (terminal blebbing) bubbles on the cell surface, apoptotic bodies, and necrotic cells were counted.

ATP content [1] was measured on a bioluminometer 8802. To this end, ATP-reagent (0.8 ml) was placed in a cuvette and background luminescence was recorded (I_{bck}). After that 50 μl BCM suspension or incubation medium were added and luminescence (I_{sample}) was measured. For calibration, standard ATP solution ($\times 10^{-6}$M, 50 μl) was than added to the cuvette and luminescence (I_{st}) was recorded. ATP content in the sample was calculated by the formula:

$$[ATP]_{sample} = \frac{V_{st}}{I_{sample}} \times \frac{V_{sample}}{I_{st}} \times [ATP]_{st},$$

where V_{st} is the volume of ATP standard, V_{sample} — sample volume, $[ATP]_{st}$ — ATP concentration in the standard solution (nmol/10^6 cells).

Lipid peroxidation (LPO) in BMC suspension was analysed by measuring the content of malonic dialdehyde (MDA) in the reaction with thiobarbituric acid (TBA) according to the standard technique (incubation with TBA, extraction with butanol, and spectrophotometry).

For evaluation of cytotoxic effects of xenobiotic, the cells were incubated with 50 μl MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide;

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Sigma; 5 mg/ml) for 1.5 h at 37°C [5]. Formazan crystals were dissolved in 500 μl 0.1 N HCl-isopropanol. Optical density of samples was measured on an SF-26 spectrophotometer at λ=570 nm, background density at λ=640 nm was distracted.

For evaluation of the *in vitro* effect of ATP on BMC, the cells were incubated with 0.1, 1, and 10 mM ATP (Reanal) for 15 min at 37°C and examined under a microscope after 5, 15, 30, 45, and 60 min. In some samples doxorubicin (10^{-6} M) was added to BMC suspension on min 15 of incubation. In *in vivo* experiments, the mice received single intramuscular injection of doxorubicin in a maximum tolerated dose (6 mg/kg in physiological saline, acute administration) or 10 daily injections of 0.95 mg/kg doxorubicin ($^1/_{10}$ LD_{50}, subacute administration).

The data were processed statistically using Student *t* test.

RESULTS

Incubation of BMC with 0.1, 1, and 10 mM ATP induced dynamic changes in the plasma membrane manifested as initial and terminal blebbing, which corresponded to *in vitro* effects of ATP on other cells [9]. ATP in low concentration (0.1 mM) induced reversible initial blebbing starting from the first minutes of incubation. ATP in higher concentration (1 mM) corresponding to ATP concentration in extracellular space in some pathological processes such as cytolysis and inflammation [13] caused maximum blebbing after 30-min incubation. Maximum ATP concentration (10 mM) produced a pronounced cytotoxic effect and induced necrosis (Table 1).

Preincubation with doxorubicin in a concentration of 10^{-6} M producing a membranotoxic effect [2] did not modulated blebbing induced by 1 mM ATP, but the number of necrotic cells increased compared to the effects of doxorubicin or ATP alone (Fig. 1).

In vivo application of doxorubicin reduced cell sensitivity to ATP and modulated blebbing. Thus, initial blebbing induced by 1 mM ATP was delayed or absent in BMC obtained from doxorubicin-treated animals (1- and 10-day doxorubicin treatment, respectively; Table 2).

Induction of oxidative stress leading to damage to cytoskeleton, membrane lipids, and proteins is the key mechanism of doxorubicin cytotoxicity [2]. Administration of doxorubicin to experimental mice increased MDA production in BMC 24 h postinjection (to 12.92±0.56 μmol/liter) and decreased it after 2 and 10 days of treatment (to 8.24±1.11 and 4.97±0.94 μmol/liter, respectively). ATP (1 mM) added to the incubation medium had no effect on MDA generation in intact BMC, but inhibited this process in BMC exposed to doxorubicin for 24 h *in vivo* and potentiated it in cells isolated from animals receiving doxorubicin for 10 days (Fig. 2). These changes probably reflect the modulatory effect of ATP on free radical generation in cells during oxidative stress.

TABLE 1. *In Vitro* Membranotoxic and Cytotoxic Activity of ATP (% of Total Cell Number) in BMC (*M*±*m*)

Experimental conditions	Incubation time, min				
	5	15	30	45	60
ATP, 0.1 mM					
initial blebbing	14.67±3.88****	3.83±2.65***	16.33±1.59**	9.83±1.34**	9.00±0.85*
terminal blebbing	0.50±0.35	1.67±0.41	2.67±0.41	2.67±0.54	0.63±0.28
apoptotic bodies	5.83±1.34	8.50±0.35*	7.33±0.54**	7.17±1.34****	7.50±0.71**
necrosis	20.83±0.20*	21.33±0.89*	21.83±1.34**	24.33±0.74*	25.00±1.65*
ATP, 1 mM					
initial blebbing	7.00±0.53*	7.80±1.35***	12.38±1.85****	10.33±1.00*	5.63±0.72**
terminal blebbing	0.83±0.27	1.08±0.26	1.40±0.27****	1.14±0.10	1.20±0.14
apoptotic bodies	3.17±0.50	3.92±0.50	5.60±0.27***	5.50±0.46**	5.70±0.76****
necrosis	19.17±1.14**	19.17±2.35**	19.83±4.13	21.67±5.94	22.67±5.02
ATP, 10 mM					
initial blebbing	11.83±2.88****	18.50±3.59***	10.33±0.89****	10.33±1.78***	10.50±0.94*
terminal blebbing	0.67±0.20	1.50±0.35	1.33±0.20****	0.67±0.20	0.67±0.20
apoptotic bodies	4.33±0.74	4.83±0.41****	6.17±0.82	6.33±1.24	7.00±0.35*
necrosis	38.00±2.15*	38.17±0.54*	39.67±2.25*	42.17±1.47*	45.17±1.24*

Note. Here and in Table 2: *p<0.001, **p<0.01, ***p<0.02, and ****p<0.05 compared to the control.

TABLE 2. Modulation of ATP Effects in BMC after Acute and Subacute Administration of Doxorubicin to Experimental Animals (% of Total Cell Number; *M*±*m*)

Experimental conditions	Incubation time, min	
	5	60
ATP, 1 mM		
initial blebbing	7.00±0.53*	5.63±0.72**
terminal blebbing	0.83±0.27	1.20±0.14
necrosis	19.17±1.14**	22.67±5.02
Doxorubicin, 24 h		
initial blebbing	17.00±1.27*	8.50±0.85*
terminal blebbing	2.38±0.36**	1.38±0.28
necrosis	17.38±1.53**	21.88±1.26**
Doxorubicin, 24 h+ATP		
initial blebbing	14.38±0.86*	10.88±1.55*
terminal blebbing	1.25±0.29	1.50±0.24
necrosis	17.88±2.06**	23.00±1.58**
Doxorubicin, 10 days		
initial blebbing	10.50±2.21***	4.83±0.89***
terminal blebbing	1.33±0.74	1.83±0.74
necrosis	24.17±1.08*	30.33±1.08*
Doxorubicin, 10 days+ATP		
initial blebbing	1.33±1.03	0.33±0.11
terminal blebbing	0.83±0.20	2.17±0.54
necrosis	20.83±3.21***	30.17±3.05**

Oxidative stress is accompanied by imbalance between reduced and oxidized nicotinamide coenzymes. Incubation of BMC with doxorubicin decreased parameters of MTT test reflecting NADPH level and mitochondrial functions [5]. Doxorubicin added to the incubation medium decreased parameters of the MTT test to 0.130±0.009 compared to 0.197± 0.010 in the control ($p<0.001$), while after acute and subacute administration of doxorubicin to experimental animals these parameters were 0.176±0.026 and 0.193±0.025, respectively.

Doxorubicin cytotoxicity is largely determined by impaired function of the mitochondrial respiratory chain, because doxorubicin acts as an alternative electron acceptor from complex I [2]. Previous *in vitro* studies showed that doxorubicin reduced ATP content in rat cardiomyocyte mitochondria [14], which agrees with our data on decreased intracellular ATP level after acute administration of this xenobiotic. At the same time, doxorubicin induces oxidative stress and stimulates production of oxygen radical, which impairs ATP synthesis in cells.

Acute and subacute administration of doxorubicin to experimental animals *in vivo* modulated the content of ATP in BMC: intracellular ATP content significantly decreased 24 and 48 h after acute doxorubicin administration and increased after 10-day subacute treatment (Fig. 3).

Thus, inhibition of ATP-induced membrane blebbing after 10-day doxorubicin treatment was associated with reciprocal changes in MDA and ATP production, which attested to impaired biological functions of ATP during oxidative stress and mitochondrial dysfunction.

The absence of ATP-induced blebbing after subacute administration of doxorubicine *in vivo* probably reflects considerable changes in physicochemical properties of cell membranes and reduced cell sensitivity to ligands. At the same time, this effect can result from cytostatic-induced intrapopulational changes in

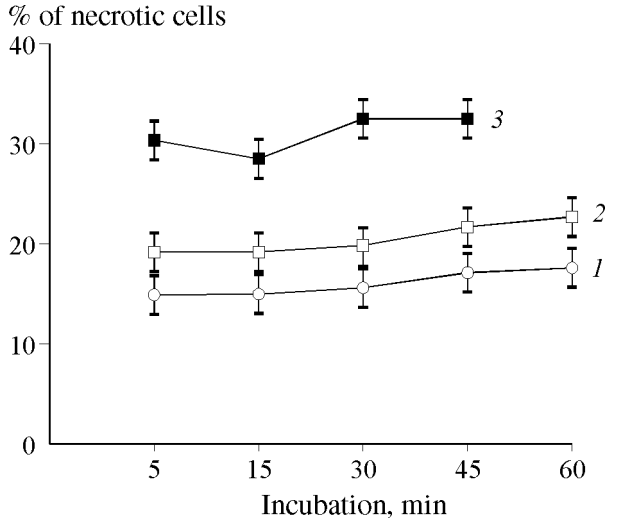

Fig. 1. Necrosis in bone marrow cells during *in vitro* incubation with 1 μM doxorubicin (*1*), ATP (*2*), and their combination (*3*).

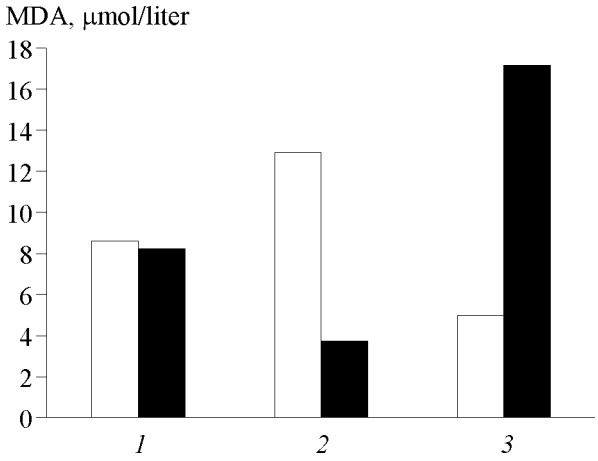

Fig. 2. *In vitro* effect of ATP on doxorubicin-induced LPO in bone marrow cells. *1*) control, *2*) doxorubicin, 24 h, *3*) doxorubicin, 10 days. Open bars: without ATP, dark bars: 1 mM ATP.

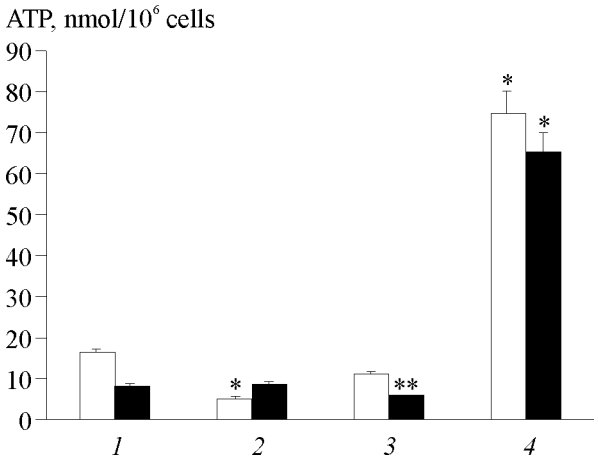

Fig. 3. ATP content in cells (open bars) and extracellular space (dark bars) during acute and subacute administration of doxorubicin *in vivo* to experimental mice. *1*) control, *2-4*) doxorubicin for 24 and 48 h and 10 days, respectively. **p*<0.001, ***p*<0.01 compared to the control.

mouse bone marrow, in particular elimination of actively proliferating cells and modulation of expression of purinergic receptors due to altered proliferative status of the cells [11]. Moreover, intracellular ATP content regulates functional activity of cytoskeleton proteins [10], while subacute administration of doxorubicin abolished blebbing-inducing effect of ATP.

Thus, ATP induces reversible membrane blebbing in BMC *in vitro*. Long-term treatment with doxorubicin, an inductor of oxidative stress and mitochondrial dysfunction, decreases BMC sensitivity to ATP.

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