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EVALUATION OF MITOGENIC EFFECTS OF OXYTOCIN ON CULTURED HUMAN LYMPHOCYTES

ĐELIĆ N.

Department of Biology, Faculty of Veterinary Medicine, University of Belgrade, Yugoslavia

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Oxytocin produces numerous biochemical effects after binding to its membrane receptors, including the modulation of mitotic activity of various cell lines. The objective of this investigation was to evaluate the effects of oxytocin on mitotic activity of cultured human lymphocytes. We analysed mitotic and proliferation indices, as well as cytokinesis block proliferation index in phytohaemagglutinin-activated human peripheral blood lymphocytes. Seven experimental concentrations of oxytocin were used in a range from 0.001 IU/ml to 0.1 IU/ml. Human recombinant insulin and N-methyl-N-nitro-N-nitrosoguanidine (MNNG) were used as positive controls. Mitotic index was not significantly influenced by oxytocin treatment. In addition, proliferation index (PI) and cytokinesis block proliferation index (CBPI) as parameters of cell cycle kinetics were not significantly changed after oxytocin treatment. Therefore, oxytocin exhibited neither stimulating, nor cytotoxic (or cytostatic) effects on cultured human lymphocytes.

Key words: cytokinesis block proliferation index, mitotic index, oxytocin, proliferation index.

INTRODUCTION

Although oxytocin is usually considered as a neurohypophyseal hormone, there is increasing evidence of the existence of peripheral oxytocinergic tissues and a possible role for oxytocin in autocrine and paracrine control (Jevremović et al., 1991; Nicholson and Pickering, 1993; Kumamoto et al., 1995; Martens et al., 1998).

It is well established that oxytocin produces various physiological effects after binding to specific membrane receptors. Oxytocin receptors are functionally coupled to GTP-binding proteins involved in activation of the phosphoinositide pathway, accompanied by elevated Ca^{2+} concentration and protein tyrosine phosphorylation (Martens et al., 1998; Sanborn et al., 1998; Berrada et al., 2000).

It has been shown that oxytocin can exert trophic effects, such as stimulation of myoepithelial cell differentiation and proliferation. A possible role in

stimulation of mitogenesis is suggested from experimental data that oxytocin causes rapid tyrosine phosphorylation of mitogen-activated protein (MAP) kinase in cultured rat myometrial cells (Nohara et al., 1996). In addition, oxytocin can replace interleukin -2 in mitogenic induction of mouse spleen T-lymphocytes caused by gamma-interferon (Johnson and Torres, 1985).

On the other hand, oxytocin can inhibit cell proliferation of various cultivated cell lines. Thus, oxytocin failed to increase DNA synthesis in cultured rat hepatocytes (Bhora et al., 1994), and even inhibited cell proliferation of human breast cancer cell lines (Sapino et al., 1998), as well as cultured human endometrial cancer cells (Cassoni et al., 2000).

Considering the fact that oxytocin influences mitotic activity of cultured cells, the objective of our investigation was to evaluate possible changes in mitotic activity of human lymphocytes exposed to oxytocin *in vitro*.

MATERIALS AND METHODS

Test substance and controls. Oxytocin (CAS No. 50-56-6 Syntocinon®, Novartis, Basel, Switzerland) was used as the test substance. Positive controls were N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (Sigma Chemical Co., St. Louis, MO) at a final concentration of 10^{-6} M, and 10^{-8} M human recombinant insulin (Humulin regular, Elli Lilly and Co., Indianapolis, IN). The negative contol was 0.9% NaCl.

Lymphocyte culture. Human peripheral blood lymphocyte cultures were prepared according to a slight modification of the protocol described by Evans and ORiordan (1975). Heparinised whole blood samples (0.8 ml) obtained from three healthy men under 35 years of age were added to vials with 9.2 ml of pre-warmed Parker 199 medium (Torlak, Belgrade, Yugoslavia) containing 30% of inactivated calf serum (Serva, Heidelberg, Germany) and 0.04 mg/ml of phytohaemagglutinin (Murex Diagnostics Ltd., Dartford, England). At the beginning of incubation 5-bromo-2'-deoxyuridine (BrdUrd, Sigma Chemical Co., St. Louis, MO) was added to each culture to obtain a final concentration of 25 μM. Cultures were incubated in the dark for 72 h at 37°C.

Treatment. Exactly 47 h and 30 min after the beginning of incubation oxytocin (Syntocinon®) was added to cultivation vials in such amounts to obtain final experimental concentrations of: 0.001, 0.002, 0.005, 0.01, 0.02, 0.05 and 0.1 IU/ml. Positive and negative controls were added in separate cultivation vials to obtain a final concentration of 10-6 M (MNNG) and 10^{-8} M (insulin).

Analysis of mitotic and proliferation indices. Two hours before harvesting, colcemid (Ciba, Basel, Switzerland) was added to the cultures to achieve a final concentration of 0.5 μg/ml. After standard chromosome preparation microscopic slides were stained according to the fluorescence-plus-Giemsa (FPG) procedure (Perry and Wolff, 1974). Mitotic index was determined on 1000 or more cells, whereas cell cycle kinetics was estimated by proliferation index (Tice and Ivett, 1985) scored on at least 200 metaphases per donor. Proliferation index was calculated according to the formula:

$$PI\ (M_1 + 2M_2 + 3M_3) / 100$$

- M_1, M_2 and M_3 refer to the percentage of metaphases in the first, second and third mitotic cycle.

Analysis of cytokinesis block proliferation index. In a separate experiment, we analysed the cytokinesis block proliferation index (CBPI) on PHA-activated human lymphocytes. The experimental protocol was the same as described above, except that RPMI 1640 (Gibco, Grand Island, NY) was used as the medium, cultures were not treated with BrdUrd, and after 47h 30 min from culture initiation, cytochalasine B (Sigma Chemical Co., St. Louis, MO) was added to the final concentration of 6 μg/ml. In addition, hypotonic treatment after 72 h of incubation was much shorter (only 3 min). After standard chromosome preparation binucleated lymphocytes were stained in 2% Giemsa (Kemika, Zagreb, Croatia) in Gurr buffer (pH = 6.8). Cytokinesis block proliferation index (CBPI) was calculated on at least 1000 cells per donor, according to the formula of Surralles et al. (1994):

$$CBPI = [M_I + 2M_{II} + 3(M_{III} + M_{IV})] / 100$$

- where M_I to M_{IV} represent the percentage of cells with one to four nuclei.

Statistical analysis. Mitotic index was analysed by Students *t*-test, whereas proliferation index (PI) and CBPI were analysed by $\chi 2$ test.

RESULTS

The values obtained for mitotic index are presented in Table 1. Although the concentrations of oxytocin higher than 0.01 IU/ml produced a slight increase of MI compared to the negative control, mitotic index (MI) did not change significantly upon oxytocin treatment. In contrast, MNNG decreased ($p<0.01$) the MI value (MI=3.05%) in comparison to the control level (MI=5.42%), while insulin was used as a positive control able to increase mitotic activity. Thus, the concentration of 10^{-8} M of human recombinant insulin, which was found to be the most efficient at increasing the MI of human lymphocytes (Djelić, 2001), caused a significant ($p<0.01$) elevation of mitotic index (MI=9.34%).

The values for proliferation index (PI) (Table 1) clearly demonstrate that oxytocin caused non-significant alterations of cell cycle kinetics compared both to the values for untreated cultures and the negative control. As for the positive controls, MNNG suppressed PI values (PI=1.50, $p<0.001$), whereas human recombinant insulin caused a significant increase (PI=1.98, $p<0.01$).

Finally, the results for cytokinesis block proliferation index (CBPI) are presented in Table 2. These results are completely consistent with the data obtained for PI in the first experiment. Likewise, CBPI values did not depart significantly from the negative control level (CBPI=2.02) after application of the examined concentrations of oxytocin. As expected, the positive control MNNG suppressed (CBPI=1.77, $p<0.001$), while insulin stimulated CBPI (CBPI=2.12, $p< 0.05$).

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Table 1. Mitotic index values and proliferative kinetics of cultured human lymphocytes treated with oxytocin

conc. of oxytocin	MI (%)	Percent cells in			PI
		M_1	M_2	M_3	
untreated	5.42	30.3	58.5	11.2	1.81
negative control	5.77	29.2	60.0	10.8	1.82
10^{-6} M MNNG	3.05**	54.8	39.8	5.3	1.50***
10^{-8} M insulin	9.34**	14.3	73.3	12.3	1.98**
0.001 IU/ml	5.64	28.7	61.8	9.5	1.81
0.002 IU/ml	5.36	30.0	61.0	9.0	1.79
0.005 IU/ml	4.97	36.2	57.3	6.5	1.70
0.01 IU/ml	6.20	26.0	64.2	9.8	1.84
0.02 IU/ml	7.05	24.2	65.2	10.7	1.87
0.05 IU/ml	5.86	31.0	61.8	7.2	1.76
0.1 IU/ml	6.15	28.3	63.3	8.3	1.80

MI - mitotic index; M_1 M_2 and M_3 refer to the percentage of cells in the first, second and third mitotic cycle.
PI- cell proliferation index
$PI = (M_1 + 2M_2 + 3M_3) / 100$ ** $p < 0.01$; *** $p < 0.001$ (χ^2 test)

Table 2 - **Cytochalasine block proliferation index (CBPI)** in cultures of human lymphocytes treated with oxytocin

conc.of oxytocin	Distribution of cells according to No of nuclei (%)			
	M_I	M_{II}	M_{III+IV}	**CBPI**
untreated	16.2	68.9	14.9	1.99
negative control	17.2	63.9	18.9	2.02
10^{-6} M MNNG	32.5	57.6	9.9	1.77***
10^{-8} M insulin	6.3	75.9	17.8	2.12*
0.001 IU/ml	20.5	63.6	15.9	1.95
0.002 IU/ml	13.7	68.4	17.9	2.04
0.005 IU/ml	19.3	66.1	14.6	1.95
0.01 IU/ml	15.8	70.9	13.3	1.98
0.02 IU/ml	11.9	72.8	15.3	2.03
0.05 IU/ml	17.0	66.4	16.6	2.00
0.1 IU/ml	22.0	65.5	12.5	1.90

M_I to M_{IV} represent the percentage of cells with one to four nuclei
CBPI = $[M_I + 2M_{II} + 3(M_{III} + M_{IV})] / 100$ * $p < 0.05$; *** $p < 0.001$ (χ^2 test)

DISCUSSION

Despite numerous experimental data concerning intracellular changes caused by oxytocin binding to its membrane receptors, its possible influence on nuclear genetic material has not been thoroughly investigated. In our previous study (Djelić et al., 1996) oxytocin did not induce cytogenetic changes in cultured human lymphocytes.

Since oxytocin may influence mitotic activity both as a stimulator (Nohara et al., 1996) or inhibitor (Sapino et al., 1998), depending on the cell type and experimental conditions, this investigation was conducted to evaluate whether oxytocin is capable of changing the mitotic activity and cell cycle kinetics of human lymphocytes under the same experimental conditions used in our previous genotoxicological characterization of oxytocin (Djelić et al., 1996). Therefore, in order to examine possible alterations in mitotic activity we monitored the mitotic index (MI, percentage of cells in mitosis), proliferation index (PI) and cytokinesis block proliferation index (CBPI). The PI and CBPI were determined in separate experiments: one was designed in the same way as a sister-chromatid exchange test, while in the other we used a procedure for cytokinesis-blocked micronucleus assay.

The results demonstrate that oxytocin was unable to influence mitotic acitvity significantly. Moreover, although analysis of cell proliferation activity is more sensitive than simple counts of cells in mitosis expressed as mitotic index, oxytocin did not change PI and CBPI values in relation to the control. Since the quality of culture media and other substances was strictly controlled to be the same in all separate experiments, it is conceivable that oxytocin exhibited neither stimulating nor cytotoxic (or cytostatic) effects on cultured human lymphocytes.

It is noteworthy that the concentration of human recombinant insulin selected as the positive control in this study (10^{-8} M) exhibited maximal biological effects at comparable concentrations: e.g. increase in malic enzyme gene expression at 0.4×10^{-8} M in rat differentiating brown adipocytes (Garcia-Jimenez et al., 1993), maximal stimulation of DNA synthesis in mouse embryonic fibroblastic 3T3-F442A preadipose cells (Tang et al., 1995) at 0.5×10^{-8} M, and rapid decrease in the amount of IGF-binding protein-1 mRNA in human HepG2 cells at 10^{-8} M (Babajko, 1995).

Overwhelming evidence indicates that oxytocin may influence subcellular events underlying enhanced DNA synthesis accompanied by an increase of cell cycle kinetics and overall mitotic activity. Carter et al. (1993) suggested a possible role for neurohypophyseal peptides (vasopressin and oxytocin) in the regulation of growth and development, as well as autocrine regulation of tumor growth. Some recent data (Cassoni et al., 1998), however, indicate the ability of oxytocin to inhibit cell proliferation of human neuroblastomas and glioblastomas by acting through specific oxytocin receptors. Furthermore, oxytocin inhibited estrogen-induced cell growth of MCF 7 and T47D human breast cancer cells (Cassoni et al., 1994). Moreover, there are experimental data showing that oxytocin failed to modulate mitotic activity. Thus, oxytocin did not increase ^{3}H-thymidine incorporation into DNA of cultured rat hepatocytes, despite the fact that vasopressin caused an increase of DNA synthesis of the same cell type (Bhora et al., 1994).

On the basis of the obtained results it can be concluded that oxytocin did not change the values of MI, PI and CBPI significantly. It should be noticed,

however, that Johnson and Torres (1985) demonstrated the ability of oxytocin efficiently to replace the interleukin 2 (IL 2) requirement for mitogenic induction of T cells by gamma-interferon in mouse spleen cultures. Although in the present study oxytocin was not capable of modulating mitotic activity alone, there is still a possibility that, had we treated the cultures simultaneously with some hormone or growth factor, we might have discovered certain stimulating effects.

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Address for corespondence:
Đelić N.
Department of Biology Faculty of Veterinary Medicine
University of Belgrade
Bul. JNA 18, 11000 Belgrade, Yugoslavia
e-mail: ndjelic@vet.bg.ac.yu

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EVALUACIJA MITOGENIH EFEKATA OKSITOCINA U KULTURAMA HUMANIH LIMFOCITA

ĐELIĆ N.

SADRŽAJ

Oksitocin dovodi do različitih biohemijskih efekata nakon vezivanja za svoje membranske receptore, uključujući modulaciju mitotske aktivnosti pojedinih ćelijskih linija. Cilj ovog istraživanja bio je da se procene efekti oksitocina na mitotsku aktivnost humanih limfocita u kulturi. Analiziran je procenat ćelija u mitozi (mitotski indeks) i proliferacioni indeksi kao pokazatalji ćelijske kinetike humanih limfocita aktiviranih fitohemaglutininom. Upotrebljeno je sedam eksperimentalnih koncentracija oksitocina u rasponu od 0.001 IU/ml do 0.1 IU/ml. Humani rekombinantni insulin i N-metil-N'-nitro-N-nitrozogvanidin (MNNG) upotrebljeni su kao pozitivne kontrole. Rezultati ukazuju da tretman kulutra limfocita periferne krvi oksitocinom ne menja značajno vrednosti mitotskih i proliferacionih indeksa, odnosno da oksitocin nije ispoljio mitogene, citotoksične ili citostatske efekte.