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CYTOGENETIC ANALYSIS OF THE EFFECTS OF EPINEPHRINE ON CULTURED HUMAN LYMPHOCYTES

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Epinephrine can modulate mitotic activity of normal and malignant cells and exhibit genotoxic potential in some test-systems. It is assumed that metabolic conversion of phenolic groups in the catechol ring of epinephrine leads to the formation of reactive derivatives and superoxide anions, capable of damaging cellular molecules including DNA. The aim of the present study was to evaluate the cytotoxic and genotoxic effects of epinephrine on human peripheral blood lymphocytes in vitro. The lowest concentration of epinephrine used in these experiments (5×10^{-10} M) was calculated to be in the range of the physiological blood level of epinephrine in humans. Three experimental concentrations corresponded to minimal (2×10^{-7} M), average (10^{-6} M) and maximal (5×10^{-6} M) therapeutic doses in human medicine. In addition, the highest concentrations exceeded the maximal therapeutic dose 10-fold (5×10^{-5} M) and 30-fold (1.5×10^{-4} M), respectively. On the basis of the results obtained it can be concluded that epinephrine had no influence on the appearance of chromosome aberrations under the described experimental conditions. However, mitotic index was significantly lower in cultures treated with the three highest concentrations of epinephrine used in this investigation.

Key words: cancer, epinephrine, genotoxicity, mitotic index

INTRODUCTION

Epinephrine has been called the hormone of "fight or flight" due to its immediate release under the influence of various stressors. Moreover, there is increasing evidence that stress hormones and neurotransmitters may represent a link between the immune, endocrine and central nervous systems. The physiological effects of epinephrine prepare the body for extraordinary physical and mental exertion. At the biochemical level, specific binding of epinephrine to adrenergic receptors coupled to heterotrimeric glycoproteins (G-proteins) initiates a cascade of biochemical and molecular responses inside the cell leading to changes in cellular activity (Sanders, 1995).

Epinephrine has the highest affinity for α -adrenoceptors. Binding of epinephrine to α -adrenoceptors activates G_s to stimulate adenylate cyclase. Pharmacological and stereochemical investigations revealed that the aromatic catechol moiety of epinephrine is essential for its agonist activity (Main and Tucker, 1985).

Although data in the literature concerning physiological and biochemical changes under the influence of epinephrine and other catecholamines in various mammalian tissues are very abundant, possible genotoxic and mutagenic effects have been evaluated only in a few studies. Thus, McGregor *et al.* (1988) examined the mutagenicity of catecholamines (including epinephrine) in the mouse lymphoma L5178Y cell assay. Although, according to their results, epinephrine expressed some mutagenic potential, it is still not clear whether these results can be confirmed in other test-systems on mammalian cells.

There are some interesting experimental data that dopamine causes substantial DNA damage manifested mainly by strand breaks and base modifications (Graham *et al.*, 1978; Moldeus *et al.*, 1983). This effect is probably caused by dopamine oxidative metabolites. Namely, it is conceivable that oxidation of dopamine phenolic groups results in the formation of superoxide anions. This hypothesis is supported by the finding that induction by L-dopamine of DNA strand breaks in human fibroblasts was inhibited by coinubation with superoxide dismutase (SOD) (Moldeus *et al.*, 1983).

The aim of the present study was to evaluate mitotic activity and possible cytogenetic changes in cultured human lymphocytes treated with a wide range of epinephrine concentrations. Experimental concentrations of epinephrine were calculated to correspond the human physiological blood level, the therapeutic range, and up to 30-fold greater than the therapeutic concentration. Thus, the results of this investigation should contribute to a better understanding of the genetic risk at a wide range of epinephrine concentrations.

MATERIALS AND METHODS

Test substance and controls. Epinephrine (CAS No., Adrenalin, Jugoremedija, Zrenjanin) was used as the test substance. An acetone solution of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (Sigma Chemical Co., St. Louis, MO) at a final concentration of 10^{-6} M and human recombinant insulin (Inutral HM-100, ICN Galenika) at concentration 10^{-8} M, were the positive controls. The negative control was prepared as a placebo in Jugoremedija (Zrenjanin) as a solution of all compounds in the preparation except the active one – epinephrine.

Lymphocyte culture. Human peripheral blood lymphocyte cultures were prepared according to a slight modification of the protocol described by Evans and O'Riordan (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). Cultures were incubated in the dark for 72 h at $37 \pm 0.5^\circ\text{C}$.

Treatment. In order to determine experimental concentrations of epinephrine comparable to the blood level of epinephrine in treated patients, we consulted a textbook on pharmacology (Reynolds, 1996). Exactly 47 h and 30 min after the beginning of incubation, epinephrine (Adrenalin, Jugoremedija) was added to cultivation vials in such amounts to obtain final experimental concentrations of: 5×10^{-10} M, 2×10^{-7} M, 10^{-6} M, 5×10^{-6} M, 5×10^{-5} M and 1.5×10^{-4} M. Positive and negative control substances were added to separate cultivation vials.

Chromosome preparation and analysis. Two hours before harvesting, colcemid (Ciba, Basel, Switzerland) was added to the cultures to achieve a final concentration of 0.5 g/ml. After standard chromosome preparation microscopic slides were stained in 10% Giemsa (Kemika, Zagreb) solution in Gurr buffer (pH 6.8). Mitotic index was determined on 1000 or more cells, whereas the chromosome aberrations were analysed on 150 metaphase spreads per experimental concentration.

In addition to the cytogenetic study, we evaluated the cytotoxic or cytostatic effects of epinephrine on cultured human lymphocytes on the same microscopic slides.

Statistical analysis. The obtained data are presented as mean \pm SE. The statistical significance of differences between control and treated groups was analysed by Student's *t*-test.

RESULTS

The results of the analysis of mitotic activity in cultures treated with epinephrine are presented in Table 1. The control values of MI were 5.98% for untreated cultures and 5.91% in cultures treated with negative control (the solvent). Epinephrine did not change the mitotic index (MI) significantly at the lower concentrations (5×10^{-10} M, 2×10^{-7} M and 10^{-6} M). However, at higher concentrations of epinephrine there was a significant decrease in MI. In cultures with a final concentration of epinephrine of 5×10^{-6} M, MI was lowered to 3.6% ($p < 0.05$), while the concentration of 5×10^{-5} M had a more profound effect decreasing MI by 55% to the value of 2.7%. Finally, at the highest experimental concentration epinephrine (1.5×10^{-4} M) decreased MI by nearly 70% in comparison to untreated cultures (MI=1.9%, $p < 0.01$). As expected, the positive controls also changed mitotic activity: the well known mutagen MNNG lowered MI to 3.4% ($p < 0.01$), whereas insulin elevated MI to 8.9% ($p < 0.01$).

The results of cytogenetic analysis are presented in Table 2. For the sake of precise comparisons, we analysed exactly 150 mitotic figures for each experimental point in control and treated cultures. Evidently, epinephrine did not influence the frequencies of observed chromosomal changes significantly. Only N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) caused a significant increase of numeric chromosome aberrations (aneuploidies and polyploidies), as well as chromosomal gaps and breaks indicating structural changes. The genotoxic effect of MNNG was most profound here as 15.3% of observed cells contained gaps and breaks. As usual, the frequency of aneuploid cells was higher than both the frequency of

polyploid cells and cells with chromosomal gaps and breaks, probably due to the unavoidable loss of chromosomes during the preparation of metaphase spreads.

Table 1. Influence of epinephrine on mitotic index of cultured human lymphocytes

Treatment	Number of cells observed	Mitotic index (%)	Percentage of control value
Untreated	2224	5.98	100.00
Negative control (solvent)	2133	5.91	98.83
10 ⁻⁶ M MNNG	2056	3.36**	59.20**
10 ⁻⁸ M insulin	2570	8.95**	149.67**
Epinephrine 5x10 ⁻¹⁰ M	2011	4.87	81.44
Epinephrine 2x10 ⁻⁷ M	2298	5.05	84.45
Epinephrine 10 ⁻⁶ M	2478	5.69	95.15
Epinephrine 5x10 ⁻⁶ M	2331	3.60*	60.20*
Epinephrine 5x10 ⁻⁵ M	2060	2.67**	44.65**
Epinephrine 1.5x10 ⁻⁴ M	1828	1.86**	31.10**

MNNG- N-methyl-N'-nitro-N-nitrosoguanidine; *p<0.05; **p<0.01; (Student's *t*-test)

Table 2. Chromosome aberration frequencies in cultured human lymphocytes treated with epinephrine

Treatment	Number of cells observed	Aneuploidies		Polyploidies		Gaps and breaks	
		No	(%)	No	(%)	No	(%)
Untreated	150	4	2.67	1	0.67	4	2.67
Negative control (solvent)	150	6	4.00	3	2.00	2	1.33
10 ⁻⁶ M MNNG	150	13	8.67*	7	4.67*	23	15.33***
10 ⁻⁸ M insulin	150	5	3.33	0	0.00	1	0.67
Epinephrine 5x10 ⁻¹⁰ M	150	5	3.33	1	0.67	2	1.33
Epinephrine 2x10 ⁻⁷ M	150	7	4.67	2	1.33	3	2.00
Epinephrine 10 ⁻⁶ M	150	4	2.67	0	0.00	1	0.67
Epinephrine 5x10 ⁻⁶ M	150	5	3.33	0	0.00	4	2.67
Epinephrine 5x10 ⁻⁵ M	150	8	5.33	1	0.67	3	2.00
Epinephrine 1.5x10 ⁻⁴ M	150	7	4.67	3	2.00	3	2.00

MNNG- N-methyl-N'-nitro-N-nitrosoguanidine; *p<0.05; ***p<0.001; (Student's *t*-test)

DISCUSSION

In the last two decades it has been clearly demonstrated that some hormones can exhibit genotoxic and mutagenic effects. Since early epidemiological investigations have shown a correlation between the prolonged use of steroid hormones and cancer (Goh, 1967), it is not surprising that steroid hormones are the best studied group. Basic mechanisms underlying the mutagenic action of natural and synthetic estrogens have been revealed. Interestingly, metabolic conversion of estrogens leads to the formation of reactive oxygen species and intermediates capable of covalently damaging DNA (Liehr, 2001). Therefore, estrogens are considered as complete carcinogens acting both as tumor promoters (increasing cell proliferation) and tumor initiators (increasing the mutation rate) (Winter and Liehr, 1996).

Despite progress in understanding the molecular mechanisms of estrogen mutagenesis, data about genotoxicologic effects of nonsteroid hormones and mediators are scarce. Since hormones are normal compounds in animal and human bodies, little attention has been paid to their significance in mutation processes (Djelić, 1997).

In this investigation we evaluated the genotoxic and cytotoxic potential of epinephrine on human lymphocytes *in vitro*. Bearing in mind that human T and B lymphocytes contain α -adrenoceptors in the plasma membrane (van Tits *et al.*, 1990), we assumed that after binding of epinephrine to α -adrenoceptors signal transduction may contribute to modulation of mitotic activity and/or the appearance of chromosomal changes.

The analysis of chromosome breakage and aberrations in this investigation, showed that epinephrine had no influence on this cytogenetic manifestation of mutagenic action. Although McGregor *et al.* (1988) observed some mutagenic action of epinephrine on mouse lymphoma L5178Y cells, they used a test for gene mutations at the thymidine kinase locus. Namely, despite this mutagenic potential of epinephrine at the gene level, the effects may be undetectable at a cytogenetic level.

Metabolic oxidation of phenolic groups of catecholamines is considered as a keystone in their genotoxic action. Reactive intermediary semiquinone radicals from dopamine cause oxidative damage to cellular components, while quinones can react with nucleophilic groups in proteins and DNA (Mason, 1979). On the other hand, free oxygen species are created during the oxidation of catechol groups, such as the superoxide anion ($O_2^{\cdot-}$). Superoxide anions appear to cause chromosome breakage and sister-chromatid exchanges in human lymphocytes *in vitro* (Emerit *et al.*, 1982). The absence of a genotoxic effect at the cytogenetic level in this study may reflect the relatively weak metabolic capacity of lymphocytes with respect to catecholamines.

To determine possible cytotoxic or cytostatic effects of epinephrine, we analysed mitotic index (MI) for each experimental concentration as well as for controls. The positive control (MNNG) decreased MI by 40%, compared to untreated cultures, possibly due to arrest of mitosis because of repair of genetic material.

Additionally, in cells with a relatively high level of genetic damage cytotoxic effects occur.

Human recombinant insulin at a concentration of 10^{-8} M was used as the positive control capable to stimulate cultured lymphocyte proliferation (Djelić and Soldatović, 1997). It should be mentioned that insulin has expressed a maximal effect at the same or very similar concentrations in a number of studies. For example, insulin increased malic enzyme gene expression at 0.4×10^{-8} M in rat differentiating brown adipocytes (Garcia-Jimenez *et al.*, 1993), maximally stimulates DNA synthesis in mouse embryonic fibroblastic 3T3-F442A preadipose cells at 0.5×10^{-8} M (Tang *et al.*, 1995), and rapidly decreases the amount of IGF-1-binding protein mRNA in human HepG2 cells at 10^{-8} M.

The available data suggest that the epinephrine-induced increase in intracellular cAMP can modulate mitotic activity of various cell types. Intracellular cAMP concentrations increase transiently prior to DNA synthesis in such different cells as bacteria, yeasts and mammalian cells (Bronstad *et al.*, 1983). Hormone-specific stimulation of adenylate cyclase elevates intracellular cAMP in regenerating rat liver hepatocytes (Boynton and Whitfield, 1981). Moreover, the growth response of hepatocytes is facilitated by α -adrenergic stimulation and subsequent increase of cAMP inside the cell (Friedman *et al.*, 1981). However, some experimental findings indicate that cAMP has dual effects on hepatocyte proliferation – stimulative effects early in the prereplicative period (G_0 and early G_1), and a marked inhibition after the transition from G_1 to S phase (Sand *et al.*, 1992). It is worth noting that during hepatocarcinogenesis α -adrenoceptors increase in number and responsiveness to isoproterenol (Refsnes *et al.*, 1986).

The interference of lymphocyte proliferation obtained in cultures treated with high concentrations of epinephrine is in accordance with the observation that stimulation of α -adrenoceptors accompanied by elevated intracellular cAMP concentration suppresses lymphocyte proliferation (Bourne *et al.*, 1974; Dell'Orco *et al.*, 1977). Moreover, Cook and McCormick (1993) have shown that cAMP interferes with mitogenic signal transduction in Rat1 cells. Likewise, cAMP inhibits growth in soft agar of mouse lung tumour cell lines (Droms, 1996).

Therefore, according to available data, cAMP can act both as a stimulator and an inhibitor of mitogenesis, depending on the cell type or phase of the cell cycle. In the present study epinephrine inhibited lymphocyte proliferation ($p < 0.05$) at the concentration 5×10^{-6} M, which is comparable to the maximal therapeutic dose in human medicine. At concentrations of 5×10^{-5} M and 1.5×10^{-4} M the inhibition was more profound ($p < 0.01$), and greater than the cytotoxic effects of the positive control.

In light of the experimental data obtained in this study it can be concluded that epinephrine did not exhibit genotoxic effects. However, high experimental concentrations of epinephrine interfere with lymphocyte proliferation *in vitro*.

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CITOGENETIČKA ANALIZA DEJSTVA ADRENALINA NA HUMANE LIMFOCITE U KULTURI

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SADRŽAJ

Adrenalin može da promeni mitotsku aktivnost normalnih i malignih ćelija. Pored toga, adrenalin ispoljava genotoksične efekte u nekim test-sistemima. Smatra se da metabolička konverzija fenolnih grupa u kateholnom prstenu adrenalina dovodi do stvaranja reaktivnih derivata i superoksidnih anjona sposobnih da oštete molekule u ćeliji, uključujući DNK. Cilj ovog istraživanja bio je da se izvrši evaluacija citotoksičnih i genotoksičnih efekata adrenalina na limfocite periferne krvi čoveka *in vitro*. Najniža eksperimentalna koncentracija adrenalina korišćena u ovom eksperimentu (5×10^{-10} M) proračunata je tako da bude upoređljiva sa fiziološkom koncentracijom adrenalina u krvi čoveka. Tri eksperimentalne koncentracije odgovaraju minimalnim (2×10^{-7} M), srednjim (10^{-6} M) i maksimalnim (5×10^{-6} M) terapijskim dozama u humanoj medicini. Pored toga, dve najviše koncentracije veće su od maksimalne terapijske doze deset (5×10^{-5} M) i trideset puta (1.5×10^{-4} M). Na osnovu dobijenih rezultata može se zaključiti da adrenalin nije značajno uticao na pojavu hromozomskih aberacija pod opisanim eksperimentalnim uslovima. Međutim, tri najviše koncentracije adrenalina upotrebene u ovom eksperimentu dovode do značajnog smanjenja mitotske aktivnosti.