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Evaluation of cytogenetic and DNA damage in human lymphocytes treated with adrenaline *in vitro*

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ABSTRACT

Catechol groups can be involved in redox cycling accompanied by generation of reactive oxygen species (ROS) which may lead to oxidative damage of cellular macromolecules including DNA. The objective of this investigation was to evaluate possible genotoxic effects of a natural catecholamine adrenaline in cultured human lymphocytes using cytogenetic (sister chromatid exchange and micronuclei) and the single cell gel electrophoresis (Comet) assay. In cytogenetic tests, six experimental concentrations of adrenaline were used in a range from 0.01–500 μM. There were no indications of genotoxic effects of adrenaline in sister chromatid exchange and micronucleus tests. However, at four highest concentrations of adrenaline (5 μM, 50 μM, 150 μM and 300 μM) we observed a decreased mitotic index and cell-cycle delay. In addition, in the Comet assay we used adrenaline in a range from 0.0005–500 μM, at two treatment times: 15 min or 60 min. In contrast to cytogenetic analysis, there was a dose-dependent increase of DNA damage detected in the Comet assay. These effects were significantly reduced by concomitant treatment with quercetin or catalase. Therefore, the obtained results indicate that adrenaline may exhibit genotoxic effects in cultured human lymphocytes, most likely due to production of reactive oxygen species.

1. Introduction

Adrenaline has been called a hormone of "fight or flight" due to its immediate release under the influence of various stressors. Actually, there is increasing evidence that stress hormones and neurotransmitters may represent a link between the immune, endocrine and central nervous systems (Kin and Sanders, 2006). The physiological effects of adrenaline prepare the body for extraordinary physical and mental exertion. It is well established that, at a molecular level, specific binding of adrenaline to membrane adrenergic receptors coupled to heterotrimeric glycoproteins initiates a cascade of biochemical responses inside the cell leading to change of cellular activity (Molenaar et al., 2007).

It has been demonstrated that adrenaline has the highest affinity for β-adrenoceptors. Binding of adrenaline to these receptors activates G_s protein to stimulate adenylate cyclase (Namiecinska et al., 2006). Pharmacological and stereochemical investigations revealed that the aromatic catechol moiety of adrenaline is essential for its agonist activity (Liapakis et al., 2004).

Although the molecular mechanisms of the effects of adrenaline and other catecholamines in various mammalian tissues are studied in detail, possible genotoxic and mutagenic effects are not investigated enough. Interestingly, it has been revealed that dopamine induces DNA strand breaks in human skin fibroblasts and gene mutations in mouse lymphoma cells (Moldeus et al., 1983). However, dopamine was not genotoxic in *Salmonella*/mammalian-microsome mutagenicity test, sex linked recessive lethal test in *Drosophila melanogaster*, sister-chromatid exchange (SCE) test in human lymphocytes and micronucleus assay in mouse and rat (Moldeus et al., 1983). In addition to these results, mutagenic effects of various catecholamines (including adrenaline) were revealed on mouse lymphoma L5178Y cell thymidine kinase locus (McGregor et al., 1988). Most likely, the molecular mechanism

Abbreviations: CAT, catalase; CBPI, cytokinesis-block proliferation index; EPI, adrenaline; FPG, fluorescence-plus-Giemsa; MN, micronucleus; ROS, reactive oxygen species; SCE, sister chromatid exchange.

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underlying the genotoxic effects of catecholamines in mouse lymphoma cells implies creation of superoxide anion (Moldeus et al., 1983; McGregor et al., 1988). In addition, there are interesting experimental findings on plasmid DNA that catechol derivatives, including adrenaline, induce DNA strand breakage by ferryl species, whereas the induction of 8-hydroxyguanine (8OHG) is due to hydroxyl radical ($OH^{\cdot}$) (Miura et al., 2000).

More recent studies have shown that noradrenaline induces primary DNA damage in the Comet assay on human lymphocytes (Djelić and Anderson, 2003) and sperm (Dobrzynska et al., 2004). Since the antioxidant enzyme catalase reduces the effect of noradrenaline in the Comet assay, it has been concluded that the DNA damage resulted mainly from reactive oxygen species (ROS). This result is in accordance with experimental findings that adrenaline and other catecholamines can be involved in redox cycling under the influence of the superoxide anion (Genova et al., 2006). Namely, adrenaline may undergo oxidation and cyclisation to adrenochrome which is reduced to the corresponding semiquinone by NADPH in liver microsomes and by NADH and mitochondrial complex I in bovine heart submitochondrial particles. Finally, the semiquinone can react with molecular oxygen therefore producing superoxide anion and regenerating adrenochrome (Genova et al., 2006).

It is worth noting that superoxide anion may induce chromosome breakage and SCEs in human lymphocytes *in vitro* (M'Bemba-Meka et al., 2007). Considering that literature data concerning the genotoxic and mutagenic effects of adrenaline are incomplete and equivocal, the aim of the present study was to evaluate genotoxic effects of a wide range of concentrations of adrenaline using two cytogenetic endpoints – SCEs and micronuclei, as well as evaluation of primary DNA damage in the single cell gel electrophoresis (Comet) assay.

2. Materials and methods

2.1. Blood samples, culture conditions and treatment

The study was approved by the local Medical Ethics Committee, performed in accordance with Declaration of Helsinki, and informed donor consent was also obtained. Human peripheral blood lymphocyte cultures were set up according to a standard protocol (Rooney and Czepulkowski, 1986). Briefly, heparinised whole blood samples (0.8 mL) obtained by venipuncture from three healthy men under 30 years of age, were added to vials with 9.2 mL of RPMI 1640 (Gibco, Grand Island, NY), supplemented with 15% of heat-inactivated foetal calf serum (Gibco, Eggenstein, Germany), 1% of antibiotics (penicillin and streptomycin, Galenika, Belgrade, Serbia) and 5 μg/ml of phytohaemagglutinin (Murex Diagnostics Ltd., Dartford, England). Duplicate cultures from each donor were incubated in the dark for 72 h at 37 °C.

In cytogenetic tests, exactly 48 h after the beginning of incubation adrenaline (Adrenaline HCl, Jugoremedija, Zrenjanin, Serbia, CAS No. 51-43-4) was added to the cultivation vials in amounts to obtain six final experimental concentrations (range from 0.01 μM to 300 μM). In order to determine experimental concentrations of adrenaline we consulted textbooks on pharmacology (Reynolds, 1996; Varagić and Milošević, 2008). Thus, in cytogenetic tests the concentration of 1 μM corresponds to average therapeutic dose of adrenaline in human medicine and 5 μM to the maximal therapeutic dose. The concentration of 0.01 μM is comparable to adrenaline plasma level during intensive stress in humans (Zouhal et al., 2008), while 50, 150 and 300 μM represent 10-fold, 30-fold and 60-fold higher doses than maximal therapeutic dose. The acetone solution of N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) (CAS No. 70-25-7, Sigma Chemical Co., St. Louis, MO) at a final concentration of 1 μM was the positive control. The negative control was prepared as placebo in Jugoremedija (Zrenjanin) as a solution of all compounds in adrenaline-HCl except an active one – adrenaline.

In the Comet assay, after the isolation on ficoll gradient, lymphocytes resuspended in RPMI 1640 were treated with appropriate concentrations of adrenaline from 0.0005 μM (comparable to physiological blood level of adrenaline in humans) to 500 μM, for 15 min or 1 h, whereas H_2O_2 as the positive control, was added to Eppendorf tubes to final concentration of 100 μM. Finally, the concentration of 300 μM of adrenaline was chosen for further co-treatment with catalase or quercetin.

2.2. Sister chromatid exchange (SCE) test

In the SCE test, in order to obtain visible SCEs 5-bromo-2′-deoxyuridine (BrdUrd, Sigma Chemical Co., St. Louis, MO, USA, final concentration 20 μM) was added in each culture one hour after the beginning of incubation. Two hours before harvesting colcemid (Ciba, Basel, Switzerland) was added to the cultures to achieve a final concentration of 0.5 μg/mL. After hypotonic treatment (0.075 M KCl) followed by three repetitive cycles of fixation in methanol/acetic acid solution (3:1, v/v), centrifugation, and resuspension, the cell suspension was dropped on chilled, grease-free coded microscopic slides, air-dried over flame, aged, and then differentially stained according to the fluorescence-plus-Giemsa (FPG) procedure (Perry and Wolff, 1974). For each donor, 60 well-spread mitoses (30 mitoses per duplicate culture) were scored, and the values obtained were calculated as SCEs per cell. At least 1000 cells per culture were scored in order to calculate the mitotic index (MI). With an aim to calculate the cell proliferation index (CPI), at least 500 metaphases per culture were examined to determine the proportion of cells in the first, second and third mitotic division (Istifli and Topaktas, 2013).

2.3. Micronucleus assay

In the cytokinesis-block micronucleus assay cytochalasin-B (Sigma Chemical Co., St. Louis, MO, USA, final concentration 6 μg/mL) was added 44 h after the beginning of incubation. After 72 h, the cells were gently rinsed in serum free RPMI 1640 medium, then exposed to short hypotonic treatment (3 min) with 0.075 M KCl at room temperature. After standard procedure of preparation (three cycles in methanol-acetic acid solution, 3:1, v/v) the staining was performed in 2% Giemsa (Merck, Darmstadt, Germany) solution in Gurr buffer (pH = 6.8). At least 1000 binucleated cells per donor were analysed for the frequency of MN at 400× magnification, according to the criteria described by Fenech (1993). The cytokinesis-block proliferation index (CBPI) was calculated according to Surallés et al. (1994).

2.4. Isolation of lymphocytes

Heparinised blood samples (4 mL) were obtained by venepuncture from three healthy male donors under 30 years of age. Lymphocytes were isolated from whole blood with Ficoll-Paque medium and centrifuged at 1900 g 15 min. The lymphocytes forming a layer were directly above Ficoll-Paque. The isolated lymphocytes were washed twice in RPMI 1640 medium, each wash was followed by a centrifugation 10 min at 1800 g. Finally, the supernatant was removed as carefully as possible without disturbing the pellet. An aliquot of 1 ml of RPMI 1640 was added and the pellet was resuspended. A manual cell count and an estimate of cell viability were performed using the Trypan blue exclusion test.