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Research article

THE INVESTIGATION OF DNA DAMAGE INDUCED BY ADRENALINE IN HUMAN LYMPHOCYTES *IN VITRO*

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Adrenaline is a neurotransmitter and hormone that plays an important role in physiological regulatory mechanisms. The objective of this study was to assess primary DNA damage in isolated human lymphocytes exposed to adrenaline using the *in vitro* comet assay. Dose-response of human lymphocytes was determined at concentration range of adrenaline from 0.01 μM to 300 μM for various treatment times (1h, 2h, 4h and 24h). The obtained results showed that adrenaline induced DNA damage at concentration range from 5 μM to 300 μM after 1h, 2h and 4h of treatment. The slightest DNA damage was observed after 24 h of adrenaline treatment - only the highest concentrations of adrenaline (150 μM and 300 μM) caused increased level of DNA damage. In order to evaluate the potential contribution of reactive oxygen species (ROS) in adrenaline-induced DNA damage we used antioxidants catalase (100 IU/mL and 500 IU/mL) and quercetin (100 μM and 500 μM). Co-treatment of lymphocytes with adrenaline (300 μM) and antioxidants for 1 h, significantly reduced the quantity of DNA in the comet tails. Therefore, it can be concluded that adrenaline exhibits genotoxic effects mainly through induction of reactive oxygen species and that some of the DNA damage is repaired during the first four hours following the treatment with adrenaline.

Key words: adrenaline, antioxidants, comet assay, human lymphocytes.

INTRODUCTION

Catecholamines are a class of chemical neurotransmitters and hormones that play an important role mainly in physiological hemodynamic regulatory mechanisms. The primary effect of the catecholamine adrenaline is physiological mobilization of resources in response to emotional and physical stress. In some animals, adrenaline is involved in temperature regulation and arousal from hybernation [1]. Adrenaline is

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also involved in the immune response via stimulating inflammatory cytokines [2]. In human medicine, adrenaline has its application in the treatment of asthma, allergic reactions, cardiac arrest and glaucoma [3]. During normal physiological conditions, there is no constant secretion of adrenaline and the basal level of adrenaline in human plasma is in a nanomolar range [4]. Under stress, excess adrenaline is released in the circulation and, after binding to adrenergic receptors, it prepares the organism for the „fight or flight" response [5,6].

Although catecholamines play a vital role under stressful conditions, excess circulating levels of adrenaline may induce detrimental effects in cells. Cardiotoxicity of adrenaline is well documented [7,8]. It was found that adrenaline stimulates proliferation of esophageal cell carcinoma via β-adrenoreceptor activation [9]. In breast cancer and adenocarcinoma adrenaline induces chemoresistance via α_2-adrenergic receptors [10,11]. Besides, the mutagenic potentials of adrenaline and dopamine have been reported in tests for gene mutations in L5178Y mouse lymphoma cells [12]. More recent studies revealed that adrenaline, noradrenaline and cortisol induce DNA damage in the comet assay on 3T3 cells [13]. However, adrenaline did not induce chromosome aberrations in cultured human lymphocytes [14].

It is assumed that catecholamines exert genotoxic effects via oxidative products formed during oxidative metabolism. In a study of Djelic and Anderson [15] catalase exhibited a protective effect against DNA damaging effect of noradrenaline, *indicating the* involment of ROS *in genotoxicity of* cathecholamine. Similarly, Miura [16] found that adrenaline and noradrenaline induce DNA strand breaks in plasmid PM2 DNA due to the creation of oxygenated derivatives of catecholamines. It is noted that oxidative DNA damage is an important mutagenic and possibly carcinogenic factor [17].

These observations prompted us to investigate the influence of adrenaline on primary DNA damage on human lymphocytes. For this purpose the effect of adrenaline was evaluated by *in vitro* comet assay. To investigate whether the mechanism underlying DNA damage of adrenaline is mediated by ROS we used antioxidants catalase and quercetin in the comet assay.

MATERIALS AND METHODS

Blood sampling and cell preparation

Peripheral blood samples (4 mL) with heparin were collected by venipuncture from two healthy male donors under 25 years of age. Whole blood was diluted 1:1 with RPMI medium, underlaid with Ficoll-Paque (Sigma) and centrifuged at 1900 g for 15 min. The lymphocyte layer (buffy coat) was washed twice in RPMI 1640 medium, each wash was followed by centrifugation for 10 min at 1800 g. The cells were suspended in a total volume of 1 ml and each reaction contained 50 μl suspension ($\approx 10^4$ cells), various amounts (μl) of the test agent and PBS buffer. The number of viable cells was determined by Trypan blue exclusion [18].

Chemical treatments

Isolated lymphocytes were treated with various concentrations of adrenaline (from concentrations corresponding to physiological values in humans (0.0005 μM) to a 60x higher concentration than the maximum therapeutic dose (300 μM) at different time intervals. The highest concentration of adrenaline (300 μM) was used for further analysis with antioxidants catalase and quercetin. Simultaneous treatment of human lymphocytes with adrenaline and the antioxidant catalase (100 IU/mL and 500 IU/mL) was conducted for 1 h and the same procedure was performed with quercetin (100 μM and 500 μM). Hydrogen peroxide (100 μM) was used as the positive control, while PBS was the negative control.

The Comet assay

Before the start of the experiment the cells were checked for viability using Trypan blue dye according to the method of Phillips [18]. Alkaline comet assay was performed according to Singh [19] and Tice *et al.* [20,21] technique with slight modifications. Briefly, after incubation with the tested compound for 1, 2, 4 and 24 h at 37°C, 100 μl of cell suspension was mixed with 100 μl of 1% low melting point agarose (LMPA). The 90 μl of suspension was rapidly pippeted onto the thin agarose layer of 1% normal melting point agarose (LMPA) spread with a coverslip, and kept at 4°C for 5 min. to solidify. After removal of the coverslip, the 90 μl of 0.5% LMPA was added, spread using a coverslip and kept at 4°C for 5 min. When the agarose was solidified, the slides were immersed in cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X–100, 10% DMSO, pH 10) overnight at 4°C. After lysis, the slides were placed in a horizontal gel electrophoresis tank and kept in freshly made cold alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13) for 30 min to allow unwinding of DNA. Electrophoresis was conducted at 4°C with 25 V and 300 mA for 30 min. After electrophoresis the slides were neutralized with Tris-HCl buffer (0.4 M, pH 7.5) for 5 min. The neutralization procedure was repeated three times. Then, the slides were fixed with cold methanol, dried and stored. Before analysis, the slides were rehydrated with ice cold distilled water and stained with 50 μl of 20 μg/ml ethidium bromide.

For visualization of DNA damage, microscope slides were examined under 400× magnification on a fluorescence microscope Olympus, CX21 (Olympus Optical Co., Gmbh Hamburg, Germany). Images of 100 randomly selected lymphocytes (50 cells from each of two replicate slides were analyzed) for each sample and the DNA damage was scored visually as described by Anderson et al. [22]. Namely, cells were graded by eye into five categories corresponding to the following amounts of DNA in the tail: (A) no damage, <5%; (B) low level damage, 5-20%; (C) medium level damage, 20-40%; (D) high level damage, 40-95%; (E) total damage, >95%. The number of comets in each sample was calculated (0 x No Migration (NM) + 1 x Low Migration (LM) + 2 x Medium Migration (MM) + 3 x High Migration (HM) + 4 x Extensive

Migration (EM)) which was referred by Collins [23] and expressed as the total comet score (TCS).

Statistical analysis

Statistical analysis was performed using analysis of variance (ANOVA) followed by Tukey's multiple test (GraphPad Software, USA). The value of total comet scores (TCS) is given as mean ± SEM. A P value of at least ≤ 0.05 is considered as statistically significant.

RESULTS

The viability of cells treated with adrenaline was greater than 90% in trypan blue exclusion test at the time of the assay. DNA damage in human lymphocytes was expressed as the TCS score. Table 1 shows the effect of adrenaline on DNA damage in human lymphocytes after different incubation times. There was a tendency of dose-dependent elevation of DNA damage in lymphocytes exposed to adrenaline. A significantly increased level of DNA damage was observed at a concentration of 5 μM - 300 μM of adrenaline compared to untreated cells after 1 hour ($P < 0.01$, $P < 0.001$). As expected cells exposed to hydrogen peroxide (100 μM) showed a high degree of DNA damage at all incubation times ($P < 0.001$). After 2 and 4 hours the value of TCS was significantly increased at doses 5-300 μM ($P < 0.05$, $P < 0.001$). The lowest DNA damage in human lymphocytes was observed at one day treatment of adrenaline, when only the highest concentrations of adrenaline (150 and 300 μM) induced significant increases level of TCS i.e. DNA damage in human lymphocytes ($P < 0.001$).

In Figure 1. The DNA damaging effect of adrenaline was presented as distribution of cells (%). It was observed that at concentration of 5-300 μM adrenaline, 18-20% of total cells were in the category D (high damage) after 1 hour. However, a weaker effect was observed after 2 hours, since 16-10% of lymphocytes were in this category (D). A similar effect was observed after 4 hour treatment of adrenaline (Table 1, Figure 1). When cells were exposed to adrenaline for 24 hours, the highest percentage of undamaged cells was in the minimum damage category (B).

The effects of catalase and quercetin on DNA damage induced by adrenaline after 60 minutes are shown in Figure 2. After one hour, catalase (100 IU/mL) in the treatment with adrenaline (300 μM) significantly reduced DNA damage in human lymphocytes ($P < 0.05$). Also, a significant decreasing trend of DNA damage was observed at 500 IU/mL of catalase ($P < 0.01$). Unlike catalase, quercetin at concentration of 100 μM did not significantly reduce the level of DNA damage caused by adrenaline ($P > 0.05$), while a protective effect of quercetin was noted at a higher concentration (500 μM) ($P < 0.01$).

Table 1. The effects of adrenaline in human lymphocytes measured by the Comet assay at different exposure times

Treatment	TCS at various incubation times			
	1h	2h	4h	24h
Negative control (PBS)	61.17±0.48	60.50±0.56	60.83±3.97	57.17±3.54
Adrenaline				
0.01 μM	62.33±0.49	61.50±0.72	61.17±1.28	56.33±0.88
1 μM	63.67±0.99	63.33±1.23	69.67±2.94	57.50±0.72
5 μM	69.33±1.02**	65.33±2.62*	75.33±3.05***	60.33±1.05
50 μM	76.33±1.41***	75.83±2.30***	92.50±2.68 ***	61.00±1.37
150 μM	95.17±1.41***	94.83±1.76***	104.50±2.78 ***	64.83±1.30***
300 μM	107.80±0.99***	108.20±1.24 ***	116.70±1.62 ***	66.67±1.12***
Positive control (H_2O_2) 100 μM	184.50±2.78***	195.50±7.28***	189.30±4.59***	189.30±4.34***

Results of the total comet score (TCS) are presented as mean values ± SE from 100 cells per experiment. Three independent experiments were performed. Statistically significant increase compared to solvent control (PBS): *P < 0.05; **P < 0.01; ***P < 0.001

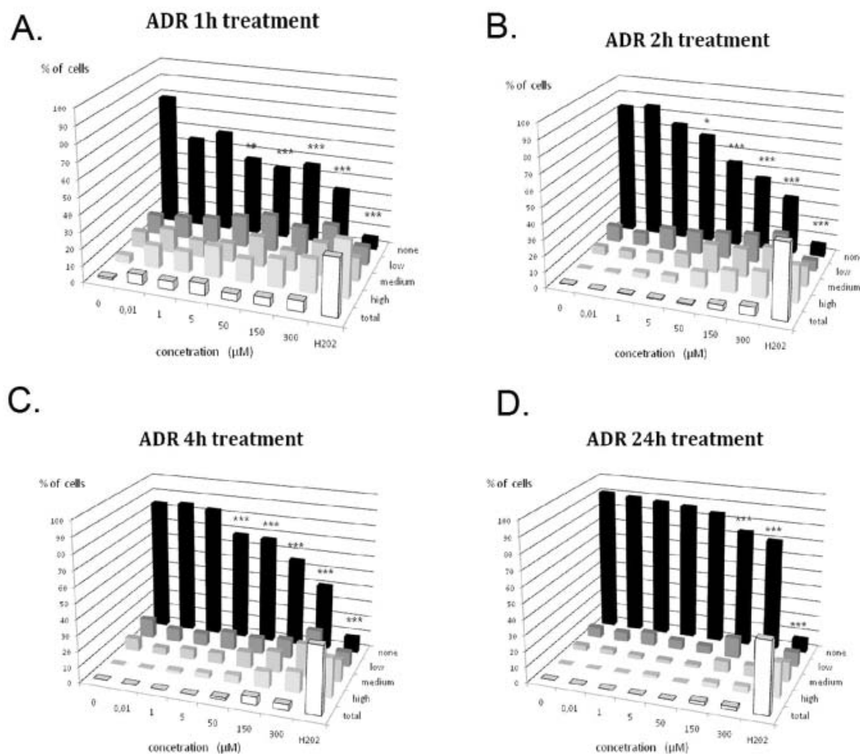

Figure 1. Distribution class of comets in human lymphocytes A. treatment with adrenaline for 1 hour, B. treatment with adrenaline for 2 hours, C. treatment with adrenaline for 4 hours, D. treatment with adrenaline for 24 hours. Statistically significant increase compared to solvent control (PBS): *P < 0.05; **P < 0.01; ***P < 0.001.

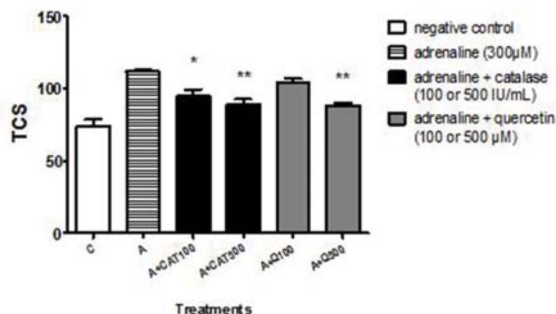

Figure 2. The effect of catalase and quercetin against DNA damage of adrenaline in human lymphocytes after 60 minutes. The results are presented as mean TCS values ± SE from 100 cells per experiment; C- negative control (PBS); A- adrenaline; A+CAT - simultaneous treatments of adrenaline and catalase (100 and 500 IU/mL); A+Q - simultaneous treatments of adrenaline and quercetin (100 and 500μM). Statistically significant increase compared to adrenaline: $^{*}P < 0.05$; $^{**}P < 0.01$

DISCUSSION

Some natural hormones can act as endogenous mutagens [24,25] and can also influence cell proliferation [26]. In addition to oestrogens, as the most studied endogenous mutagens, thyroid hormones and catecholamines may induce oxidative stress favoring the processes of mutagenesis.

The catecholamines have always attracted the attention of the scientific community due to their importance in physiological and pathological processes. Since adrenaline is an endogenous substance, it has not been considered as a possible mutagen. Hence, the data concerning evaluation of mutagenic effects of adrenaline are scarce. In this work we investigated the level of primary DNA damage in isolated human lymphocytes exposed to adrenaline using the *in vitro* comet assay.

We observed that adrenaline induced a significant increase of the TCS values at concentrations of 5 μM (maximum therapeutic dose) and higher applied concentrations compared to the negative control after 1 h, 2 h and 4 h of treatments. However, after 24 h treatment, only the highest concentrations of adrenaline (150 and 300 μM) induced significant DNA damage. This signifies that increased DNA migration detected at shorter incubation times in the comet assay could be due to strand breaks still unrepaired by excision repair [27]. We suppose that cells have repaired the most of DNA damage after 24 hours which resulted in reduced genotoxic effects of adrenaline.

Although a percentage of comets might have resulted as a consequence of early apoptosis, the comet assay is regarded as a specific test for genotoxicity. Namely, the results in the comet assay are not necessarily caused by concomitant processes leading to apoptosis [28]. Actually, DNA breakage detected in the comet assay is mainly caused by mutagen-induced DNA damage [29].

These results suggest that adrenaline induces DNA damage in human lymphocytes which is consistent with the findings of Flint *et al.* [13] that stress hormones, including adrenaline, induced DNA damage in murine 3T3 cell in the comet assay. Miura *et al.* [16] also indicated on the genotoxic potential of adrenaline. However, Djelic *et al.* [14] reported the absence of clastogenic effects of adrenaline in human lymphocytes using an *in vitro* cytogenetic test. The lack of clastogenic activity indicates that adrenaline induced DNA damage detected in the comet assay only at the molecular level. This assumption is in agreement with findings of Lankoff *et al.* [30] that microcystin-LR induced DNA breaks in the comet assay, but was unable to induce chromosome aberrations.

The catecholamines can be involved in the redox cycling accompanied by production of ROS [31,32,33]. Therefore, we used antioxidants catalase and quercetin to examine whether DNA damaging effects of adrenaline is mediated by ROS. Our results show that catalase (100IU/mL and 500 IU/mL) and quercetin (500 μM) in co-treatment with adrenaline significantly reduced DNA damage in lymphocytes. However, quercetin at 100 μM did not significantly reduce DNA damage. We assume that lower doses of quercetin in human lymphocytes are not high enough to reduce genotoxic effects, and possibly quercetin is also metabolized to the less effective form [34]. Protective effect of antoxidants used in our investigation indicated the involvement of free radicals in the genotoxic effect of adrenaline.

The contribution of ROS in genotoxicity of cathecholamines is supported by investigation of Djelic and Anderson [15] showing that catalase reduced DNA damaging effect of noradrenaline in isolated human lymphocytes. Also, Miura *et al.* [16] showed that catalase exhibited a protective effect against genotoxicity of adrenaline and noradrenaline on plasmid DNA.

DNA damaging effect of adrenaline detected in the comet assay is likely to be due to oxidative products of adrenaline. In this regard, oxidation products of adrenaline have been described in the heart, skeletal muscle, liver and blood [35,36]. Because of the unstable nature of the catechol group, catecholamine can be easily oxidized to reactive quinone and semiquinone producing ROS. Superoxide anion (O^{2-}) generated by oxidative metabolism of adrenaline can be converted to hydroxyl radical ($OH^{\bullet}$) and induce DNA breaks [37]. Also O^{2-} may promote futher oxidation of adrenaline and increase ROS production [31]. It was found that O^{2-} can reduce the activity of antioxidant enzymes such as catalase, glutathione peroxidase and NADH dehydrogenase [38]. On the other hand, quinone formed during the adrenaline oxidation process [39] can form glutathionyl adducts [40]. GSH conjugates were found to covalently bind with DNA and generate apurinic sites, which can lead to mutations [41].

It is noteworthy that we detected DNA damage of adrenaline at a concentration which is several times higher than the normal physiological levels in human blood. However, markedly elevated concentrations of circulating catecholamines are found in ischemia [42, 43], pheochromocytoma [44] and drug abuse [45]. The obtained results indicate

that applied concentrations of adrenaline (5-300 μM) established a redox cycle that multiplies ROS production. Under this condition antioxidative enzymes do not have the ability to protect cells against excess level of ROS attack which leads to oxidative stress. There is ample evidence which suggest the strong association of oxidative stress, genetic instability and cancer development [46]. ROS have the ability to cause mutations in the proto-oncogenes and thus create the possibility to be involved in malignancy [47,48].

An elevated concentration of adrenaline occurs in acute stress and can be further increased during chronic stress [49,50]. Experimental findings of DNA damaging effects of adrenaline are significant, because there is evidence that stress hormones induce cell transformation via signal transduction pathway [13]. Namely, stress hormones induce DNA damage, preventing the entry of cells in apoptosis and cell cycle arrest contributing to the transformation of cells. In support of this it is established that stress increases oxidative damage and affects the processes of DNA repair in cells [51,52]. On coherence between adrenaline and oxidative stress pointed Pereira *et al.* [53]. Their findings pointed that adrenaline regulates the activities of catalase and glutathione peroxidase, but the exact mechanism remains to be determined.

Taken together, these results suggest that adrenaline has the ability to affect genomic stability and *further* research should, *therefore* be undertaken to fully *understand* the effects of adrenaline on genetic integrity of cells.

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ISPITIVANJA OŠTEĆENJA DNK IZAZVANIH ADRENALINOM U LIMFOCITIMA ČOVEKA *IN VITRO*

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Adrenalin je neurotransmiter i hormon koji ima važne uloge u fiziološkim regulatornim mehanizmima. Predmet istraživanja u ovom radu bila je evaluacija primarnih oštećenja DNK na izolovanim humanim limfocitima izloženim dejstvu adrenalina pri-

menom *in vitro* Komet testa. Odnos doza-odgovor na limfocitima čoveka određen je u rasponu koncentracija adrenalina od 0.01 μM do 300 μM pri različitim dužinama tretmana (1h, 2h, 4h i 24h). Dobijeni rezultati pokazuju da adrenalin indukuje oštećenja DNK u rasponu od 5 do 300 μM nakon 1, 2 i 4 h tretmana. Najmanje oštećenje DNK zapaženo je nakon 24 h – samo su najveće koncentracije adrenalina (150 μM i 300 μM) prouzrokovale povećan stepen oštećenja DNK. Da bi odredili moguć doprinos reaktivnih kiseoničnih vrsta (ROS) u nastanku oštećenja DNK pod dejstvom adrenalina, koristili smo antioksidanse katalazu (100 IU/mL i 500 IU/mL) i kvercetin (100 μM i 500 μM). Kotretman limfocita adrenalinom (300 μM) i antioksidansima u trajanju od 1 h, značajno je smanjio količinu DNK u repovima kometa. Prema tome, može se zaključiti da adrenalin ispoljava genotoksične efekte uglavnom preko stvaranja reaktivnih kiseoničnih vrsta, a jedan deo oštećenja DNK se popravi tokom prva četiri časa, nakon tretmana adrenalinom.