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Assessment of adrenaline-induced DNA damage in whole blood cells with the comet assay

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Harmful effects of elevated levels of catecholamines are mediated by various mechanisms, including gene transcription and formation of oxidation products. The aim of this study was to see whether the molecular mechanisms underlying the damaging action of adrenaline on DNA are mediated by reactive oxygen species (ROS). To do that, we exposed human whole blood cells to 10 µmol L^{-1} adrenaline or 50 µmol L^{-1} H_2O_2 (used as positive control) that were separately pre-treated or post-treated with 500 µmol L^{-1} of quercetin, a scavenger of free radicals. Quercetin significantly reduced DNA damage in both pre- and post-treatment protocols, which suggests that adrenaline mainly acts via the production of ROS. This mechanism is also supported by gradual lowering of adrenaline and H_2O_2-induced DNA damage 15, 30, 45, and 60 min after treatment. Our results clearly show that DNA repair mechanisms are rather effective against ROS-mediated DNA damage induced by adrenaline.

KEY WORDS: *DNA repair; hydrogen peroxide; genotoxicity; quercetin; ROS*

The primary role of adrenaline is to mobilise resources in response to emotional and physical stress. Apart from its primary effect, adrenaline regulates insulin secretion (1) and stimulates platelet aggregation (2). It is used to treat asthma, cardiac arrest, and anaphylaxis (3). It has also been reported for cardiotoxicity (4), oesophageal cancer cell proliferation, and resistance to chemotherapy in breast cancer (5, 6). Excess adrenaline in the circulation under stress can increase blood glucose levels, blood pressure, and heart rate (7, 8) and may induce myocardial cell damage (9).

Furthermore, there are indications that the catechol group of dopamine, noradrenaline, and adrenaline is involved in the redox cycle that leads to the formation of reactive oxygen species (ROS) (10-12). Recent experimental evidence has shown the ability of adrenaline to make changes in the DNA (13-15). Some studies (10, 16, 17) suggest that oxidation and cyclisation of adrenaline result in the formation of by-products such as ROS, which may cause DNA damage.

However, studies investigating possible mechanisms of adrenaline genotoxicity are scarce and their findings ambiguous. Some confirm adrenaline-induced DNA breaks (14) and significant cell-cycle delays needed for the cell to repair genetic damage (18) but others report no association between adrenaline and chromosome aberrations in human or chicken peripheral blood lymphocytes (19, 20) or other cytogenetically detectable effects (18).

The aim of our study was to look deeper into the genotoxic action of adrenaline by focusing on the cell capacity to activate DNA repair mechanisms for damages caused by this stress hormone. To do that, we used the comet assay to quantify and analyse primary DNA damage and repair capacity in individual cells.

MATERIALS AND METHODS

Blood sampling

Whole blood samples were taken with a finger prick from three healthy female volunteers (aged between 20 and 40 years) and collected in heparinised containers. The volunteers did not smoke, drink alcohol, or receive any medicines or dietary supplements. They gave informed consent and the study was approved by the Ethics Committee of the Faculty of Pharmacy, Belgrade, Serbia (Approval No. 1103/2).

Study design

Adrenaline (CAS No. 51-43-4, epinephrine) was obtained from Sigma-Aldrich Chemie (St. Louis, MO,

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USA). Hydrogen peroxide (H_2O_2, CAS No. 7722-84-1), known to induce chromosomal aberrations, gene mutations, and DNA damage (21, 22), was obtained from ZORKA Pharma (Šabac, Serbia) and used as positive control. For negative control, cells were incubated with phosphate buffered saline (PBS, Fisher Scientific, Pittsburgh, PA). The effects of adrenaline and H_2O_2 were investigated separately in our earlier research (14) at four concentrations (5, 10, 50, and 150 µmol L^{-1} for adrenaline and 5, 10, 25, and 50 µmol L^{-1} for H_2O_2) that corresponded to the ones used in other studies of adrenaline-induced DNA damage (12, 18). For this study we selected the concentrations of 10 µmol L^{-1} of adrenaline and 50 µmol L^{-1} of H_2O_2, because they produced significant DNA damage in human whole blood cells without significantly affecting cell viability.

In order to ascertain the formation of ROS as the presumed mechanism underlying adrenaline-induced DNA damage, we also treated the cells with quercetin, a well-known scavenger of free radicals, in the concentration of 500 µmol L^{-1} taken from literature data (19, 23). It was also the most effective concentration in our recent study (24).

Two protocols were used to evaluate the contribution of different mechanisms underlying adrenaline genotoxicity: In the pre-treatment protocol, the cells were incubated with quercetin at 37 °C for 30 min prior to their exposure to adrenaline (at 37 °C for 30 min). In the post-treatment protocol, the cells were treated with quercetin under the same conditions after their exposure to adrenaline. The same experiment was conducted with quercetin and H_2O_2 following the same protocols. In all experiments, cell preparations were also incubated with PBS or adrenaline/ H_2O_2, which served as negative or positive control.

An additional set of experiments was conducted to identify the repair kinetics of adrenaline- and H_2O_2-induced DNA damage. Following adrenaline (10 µmol L^{-1}) treatment at 37 °C for 30 min, the cells were treated with PBS at 37 °C for 15, 30, 45, and 60 min. Positive control cells were exposed to 50 µmol L^{-1} H_2O_2 and then treated with PBS in the same way as described for adrenaline.

Single cell gel electrophoresis assay (comet assay)

The alkaline version of the comet assay was performed as described by Singh et al. (25). Before the treatments, 6 µL of freshly obtained whole blood samples were suspended in 0.67 % low melting point agarose (LMP, Sigma-Aldrich, St. Louis), pipetted onto slides precoated with a layer of 1 % normal melting point agarose (Sigma-Aldrich, St. Louis), and left at 4 °C for 5 min to solidify. The cell suspensions were then treated as described above. After treatment, the samples were covered with the 0.5 % LMP agarose and also left at 4 °C for 5 min, after which time they were submerged in freshly prepared and cooled lysing solution (2.5 mol L^{-1} NaCl, 100 mmol L^{-1} EDTA, 10 mmol L^{-1} Tris, 1 % Triton X 100, and 10 % dimethylsulfoxide, pH 10, adjusted with NaOH), and left to stay at 4 °C overnight. The next day, the slides were electrophoresed at 24 V and 300 mA, washed with neutralisation buffer and distilled water, and then stained with ethidium bromide (20 µg L^{-1}). The comets were scored on an Olympus BX 50 microscope (Olympus Optical Co., GmbH, Hamburg, Germany) equipped with an HBO mercury lamp (50 W, 516-560 nm, Carl Zeiss Microscopy, Jena, Germany) at 100x magnification.

DNA damage was evaluated according to Anderson et al. (22). Comets were visually scored and classified into five categories corresponding to the extent of DNA migration: (A) no damage, <5 %; (B) low level damage, 5-20 %; (C) medium level damage, 20-40 %; (D) high level damage, 40-95 %; and (E) total damage, >95 %. One hundred randomly selected nucleoids were counted on each of two slides per treatment. The DNA damage was characterised as the total DNA migration over 5 % (B + C + D + E comet classes), and the mean value was calculated for 200 nucleoids (100 per slide) for each experiment in triplicate. Apoptotic and necrotic cells were set apart from normal cells and excluded from the analyses following the instructions given by Singh (26).

Statistical analysis

We used one-way analysis of variance (ANOVA) with Tukey's *post-hoc* test to compare treatments and respective controls. Data were expressed as mean ± standard error of the mean (SEM) of three measurements. Differences at $p<0.05$ were considered statistically significant.

RESULTS

Both adrenaline and H_2O_2 caused significant primary DNA damage (Figure 1). Adrenaline induced DNA damage in around 20 % and H_2O_2 in around 30 % of the cells. The latter also induced a higher level of DNA damage (54.1 % of the cells had category D+E damage) than adrenaline (20.1 % with D+E damage). Quercetin, in turn, attenuated their damage significantly with both protocols (Figure 1) and was more effective in post-treatment, but the difference between the protocols was not statistically significant.

Figure 2 shows DNA damage repair in cells over time. While adrenaline-induced DNA damage was reduced gradually, and the reduction reached the level of significance at 45 and 60 min, H_2O_2-induced damage repair reached significance earlier, at 30 min, but then rose again, still maintaining the level of significance. In fact, DNA damage increased at 60 min in both treatments.

DISCUSSION

This study has confirmed the damaging effect of 10 µmol L^{-1} adrenaline on DNA in human whole blood cells. Quercetin (500 µmol L^{-1}) added before or after treatment

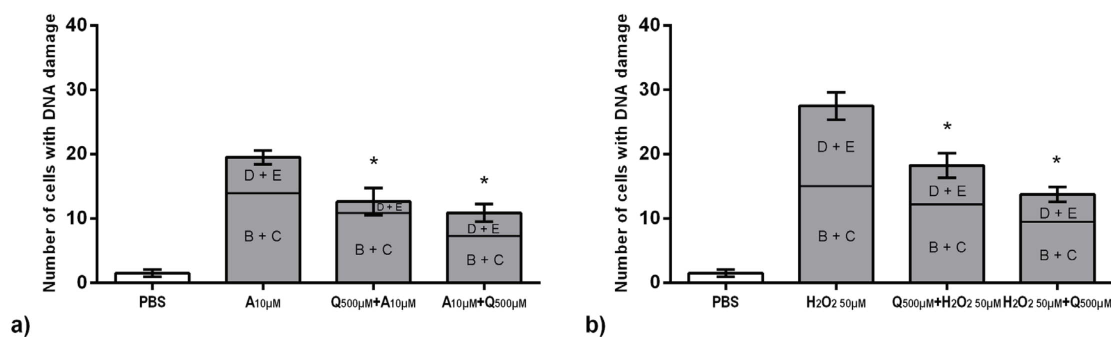

Figure 1 *a) Protective effect of quercetin (Q) against DNA damage induced by a) adrenaline (A) and b) by H_2O_2 Q+A - pre-treatment protocol; A+Q – post-treatment protocol; Q+H_2O_2 – pre-treatment protocol; H_2O_2+Q – post-treatment protocol. Bars represent the mean number of cells with DNA damage ±SEM; B+C – low and medium damage; D+E – high and total damage; * $p<0.05$* vs. *adrenaline- and H_2O_2-treated control*

with adrenaline (10 µmol L^{-1}) or H_2O_2 (50 µmol L^{-1}) significantly reduced DNA damage. It has already been reported to protect against DNA strand breaks by acting as a metal chelator (27) and by increasing the concentration of enzymatic and non-enzymatic components of the antioxidant cell system (28, 29). In our study it displayed higher efficiency in the post-treatment protocol against both oxidants. Since its most pronounced feature is the scavenging of superoxide anions, peroxy nitrite, and singlet oxygen and •OH radicals (23, 30, 31), this finding confirms that the main mechanism of adrenaline action is through free radicals. That ROS is the mechanism of adrenaline genotoxicity is also supported by the reports of antioxidant prevention of adrenaline-induced DNA damage (14, 15, 32).

As for DNA repair, it significantly reduced damage 45 and 60 min into adrenaline exposure, which is in accordance with Djelić et al. (18), where DNA damage significantly dropped after 1 h. These findings suggest that adrenaline genotoxicity decreases over time and that one hour is sufficient for cells to activate DNA repair mechanisms and compensate for the damage (33, 34). This repair was significantly effective even earlier with H_2O_2, probably because peroxide caused greater damage. Cells tend to recover faster from the impact of a more potent oxidant by activating their repair mechanisms earlier (35). Another reason for less efficient repair with adrenaline is that oxidation caused by high concentrations of adrenaline takes the quinone pathway, which yields larger amounts of highly reactive intermediates, which, in turn, slow down DNA repair (36) and prolong cell recovery.

One interesting finding in our study is that DNA repair from the effects of adrenaline weakened at 60 min. Reasons may be several. First, the concentration of adrenaline (10 µmol L^{-1}) was high enough to trigger a redox cycle, multiplying the production of ROS. Under these conditions, antioxidative enzymes cannot protect the cells against excess ROS (15). This is also confirmed by our finding of the stronger effect of quercetin in the post-treatment protocol. Second, ROS dominantly causes single- and double-stranded DNA breaks, and it takes longer for repair to compensate for double-stranded breaks (37). The resulting imbalance can also cause other types of primary DNA lesions, and overcome cell repair capacity (37, 38). Third, stress hormones induce DNA damage that prevents cell cycle checkpoint activities and the onset of apoptosis.

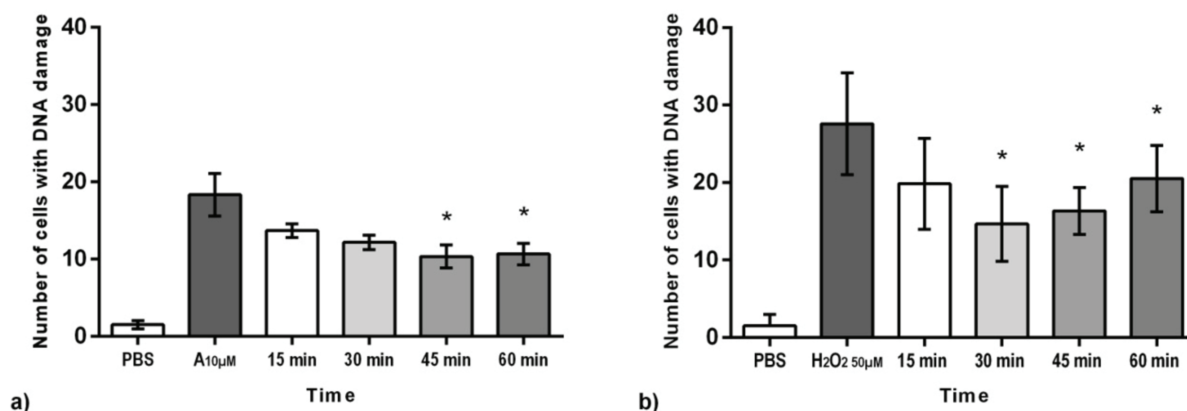

Figure 2 *DNA damage repair in cells exposed to a) adrenaline (A) and b) H_2O_2 at 15, 30, 45, and 60 min after treatment. Bars represent the mean number of cells with DNA damage (B+C+D+E comet classes) ± SEM; * $p<0.05$* vs. *adrenaline- and H_2O_2-treated control*

Stress increases ROS-generated damage and affects the DNA repair (39).

The similar responses of adrenaline and H_2O_2 to quercetin, similar DNA damage repair, as well as the stronger effect of quercetin when given after either of the oxidants, support our hypothesis that ROS production is the underlying mechanism of adrenaline genotoxicity. Highly reactive intermediates that arise from oxidised adrenaline, triggered by stress, weaken DNA repair. Even so, our results clearly show that DNA repair mechanisms are rather effective against ROS-mediated DNA damage induced by adrenaline.

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Ocjena oštećenja DNA uzrokovanog adrenalinom u stanicama pune krvi pomoću komet-testa

Štetni učinci povišenih razina katekolamina posredovani su različitim mehanizmima, uključujući transkripciju gena i formiranje produkata oksidacije. Svrha je ovoga istraživanja bila utvrditi jesu li molekularni mehanizmi koji stoje u osnovi štetnoga djelovanja adrenalina na DNA posredovani reaktivnim vrstama kisika (ROS). Da bismo to postigli, izložili smo humane stanice pune krvi adrenalinu (10 μmol L^{-1}) i vodikovu peroksidu (50 μmol L^{-1}) (pozitivna kontrola), koje su odvojeno predtretirane i post-tretirane kvercetinom (500 μmol L^{-1}), „hvatačem" slobodnih radikala. Kvercetin je značajno smanjio broj stanica s DNA oštećenjima i u predtretmanu i u post-tretmanu, što je sugeriralo da adrenalin djeluje uglavnom stvaranjem ROSa. Taj je mehanizam podržan i postupnim smanjenjem broja stanica s oštećenjima DNA izazvanim adrenalinom i H_2O_2 u vremenskim intervalima od 15, 30, 45 i 60 minuta nakon tretmana. Naši rezultati jasno pokazuju da su mehanizmi popravka prilično učinkoviti u odnosu na ROS-posredovana oštećenja DNA izazvana adrenalinom.

KLJUČNE RIJEČI: *genotoksičnost; kvercetin; popravak DNA; ROS; vodikov peroksid*