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Communication

Free radical scavengers can modulate the DNA-damaging action of alloxan★✪

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Alloxan can generate diabetes in experimental animals and its action can be associated with the production of free radicals. It is therefore important to check how different substances often referred to as free radical scavengers may interact with alloxan, especially that some of these substance may show both pro- and antioxidant activities. Using the alkaline comet assay we showed that alloxan at concentrations 0.01–50 μM induced DNA damage in normal human lymphocytes in a dose-dependent manner. Treated cells were able to recover within a 120-min incubation. Vitamins C and E at 10 and 50 μM diminished the extent of DNA damage induced by 50 μM alloxan. Pre-treatment of the lymphocytes with a nitrone spin trap, α-(4-pyridil-1-oxide)-*N*-t-butylnitrone (POBN) or ebselen (2-phenyl-1,2-benzisoselenazol-3(2*H*)-one), which mimics glutathione peroxides, reduced the alloxan-evoked DNA damage. The cells exposed to alloxan and treated with formamidopyrimidine-DNA glycosylase (Fpg) and 3-methyladenine-DNA glycosylase II (AlkA), enzymes recognizing oxidized and alkylated bases, respectively, displayed greater extent of DNA damage than those not treated with these enzymes. The results confirmed that free radicals are involved in the formation of DNA lesions induced by alloxan. The results also suggest that alloxan can generate oxidized DNA bases with a preference for purines and contribute to their alkylation.

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Abbreviations: AlkA, 3-methyladenine-DNA glycosylase II; AP-site, apurinic or apyrimidinic site; Endo III, endonuclease III; Fpg, formamidopyrimidine DNA-glycosylase; MNNG, *N*′-methyl-*N*-nitrosoguanidine; POBN, α-(4-pyridie-1-oxide)-*N*-t-butylnitrone.

Alloxan (2,4,5,6-tetraoxypyrimidine; 5,6-dioxyuracil) is known to induce diabetes in experimental animals and its action can induce several processes: oxidation of –SH groups, inhibition of glucokinase, generation of free radicals and disturbances in calcium homeostasis (Szkudelski *et al.,* 1998). In pancreatic β-cells alloxan is reduced in the presence of reducing agents such as reduced glutathione (GSH), cysteine and protein-bound sulfhydryl groups or NADH (Donnini *et al.*, 1996; Lenzen & Munday, 1991; Murata *et al.*, 1998). Reduction of alloxan generates dialuric acid which can be re-oxidized back to alloxan forming a redox cycle for the generation of superoxide radicals in the presence of reducing agents. These radicals and their products can damage DNA. Therefore, the level of DNA damage induced by alloxan can be determined by the use of substances interacting with free radicals. Some of these substances, including vitamins, may display pro- or antioxidant activities, depending on the extra- and intracellular conditions.

In this study, we investigated DNA damage evoked by alloxan in normal human peripheral blood lymphocytes using the alkaline comet assay. To search for the mechanisms of this effect we checked the efficacy of various free radical scavengers: vitamins C and E, ebselen (2-phenyl-1,2-benzisoselenazol-3(2*H*)-one) and the spin trap α-(4-pyridil-1-oxide)-*N*-t-butylnitrone (POBN) in modulating the DNA-damaging potential of alloxan. To determine the role of oxidative DNA damage in alloxan genotoxicity, we employed endonuclease III (Endo III) and formamidopyrimidine DNA-glycosylase (Fpg). Endo III converts oxidized pyrimidines into strand breaks, which can be detected by the comet assay (Collins *et al.*, 1993). Fpg is involved in the first step of base excision repair to remove specific modified bases from DNA to create apurinic or apyrimidinic site (AP-site) which is subsequently cleaved by its AP lyase activity giving a gap in the DNA strand, which can be detected by the comet assay (Evans *et al.,* 1997). To check the alkylating potential of alloxan we used 3-methyladenine-DNA glycosylase II (AlkA) which has a broad substrate specificity including alkylated purines, O^2-alkylpyrimidines and hypoxanthine (Tudek *et al.,* 1998; Gasparutto *et al.,* 2002).

MATERIALS AND METHODS

Endo III, Fpg and AlkA were gifts from Drs. B. Tudek and J. T. Kuśmierek of the Institute of Biochemistry and Biophysics, Polish Academy of Sciences (Warsaw, Poland). Lymphocytes were isolated from human peripheral blood by centrifugation in a sucrose density gradient. Alloxan was added to a suspension of the lymphocytes in RPMI 1640 medium to give a final concentration in the range 0.1–1000 μM. Lymphocytes were incubated with alloxan for 1 h at 37°C. Each experiment included a positive control, which was hydrogen peroxide at 10 μM applied for 10 min at 4°C. Ebselen at a final concentration of 50 μM and POBN at 100 μM were added to the cells along with alloxan. In experiments with vitamins the cells were preincubated for 30 min with either of them at 10 or 50 μM, washed and incubated with alloxan. To examine DNA repair, the cells after the treatment with alloxan, as well as control samples, were washed and resuspended in fresh, drug-free RPMI 1640 medium preheated to 37°C. Aliquots of the suspension were taken immediately and 30, 60 and 120 min later. Comet assay was performed under alkaline conditions essentially according to the procedure of Singh *et al.* (1988) with some modifications (Klaude *et al.,* 1996) as described previously (Blasiak & Kowalik, 2000). In the repair enzymes treatment the samples after lysis were washed three times in an enzyme buffer containing 40 mM Hepes/KOH, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml bovine serum albumin, pH 8.0. Aliquots of 30 μl of Endo III or Fpg at 1 μg/ml in the buffer were applied to the cells in agarose on slides, and incubation for 30

min at 37°C was performed (Collins *et al.*, 1993; Evans *et al.*, 1995). When AlkA was employed the buffer contained 70 mM Hepes/KOH, 1 mM EDTA and 1 mM β-mercaptoethanol, pH 7.6. To check the ability of the enzymes to recognize oxidized or alkylated DNA bases in our experimental conditions, lymphocytes were incubated with H_2O_2 or *N*′-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) and post-treated with a suitable enzyme. To assess the magnitude of DNA modification introduced by alloxan and recognized by an enzyme, we compared the differences in DNA damage between enzyme-treated and untreated groups in the presence and in the absence of alloxan. Additionally, we compared DNA damage observed after treatment with an enzyme in the presence and in the absence of alloxan. The objects were observed at 200 × magnification in an Eclipse fluorescence microscope (Nikon, Japan) attached to a COHU 4910 video camera (Cohu Inc., U.S.A.) equipped with a UV-1A filter block and connected to the personal computer-based image analysis system Lucia-Comet v. 4.51 (Laboratory Imaging, Czech Republic). Fifty images were randomly selected from each sample and the tail DNA (%) was measured. Two parallel tests with aliquots of the same sample of the cells were performed for a total of 50 cells and the mean tail DNA was calculated. The tail DNA is positively correlated with the level of DNA breakage in a cell (Singh *et al.*, 1988). The mean value of the tail DNA in a particular sample was taken as an index of DNA damage in the sample. All the values in this study were expressed as means ± S.E.M. from two separate experiments, i.e. data from two experiments were pooled and the statistical parameters were calculated. The differences between variations were calculated by Fisher-Snedecor test. If no significant differences were found, the differences between the means were evaluated by applying the Student's *t*-test. Otherwise, the Cochran-Cox test was used.

RESULTS AND DISCUSSION

Figure 1 shows that DNA damage in the lymphocytes exposed for 1 h at 37°C to alloxan measured as comet tail DNA increased with the increase of alloxan concentration up to 200 μM and was constant at higher concentrations of the drug. The cells exposed to 50 μM alloxan were able to repair their DNA during a 120-min repair incubation (data not shown). The DNA repair enzymes Fpg and AlkA recognized more DNA damage in the lymphocytes

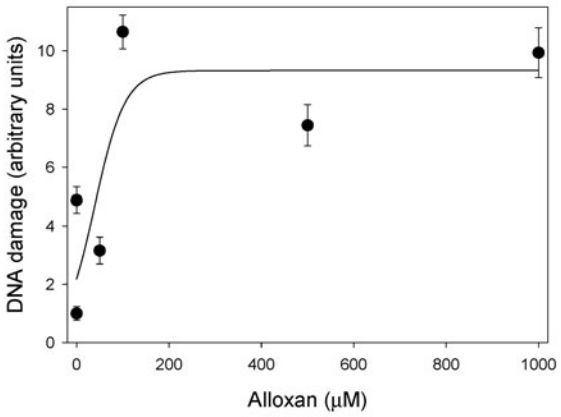

Figure 1. DNA damage measured by comet tail DNA in human lymphocytes exposed to alloxan for 1 h at 37°C.

The figure shows mean results from three independent experiments. The number of cells analyzed in each treatment was 100; error bars denote S.E.M. Regression line was calculated by means of the least square method; the *R* value was equal to 0.844; $P < 0.001$ for all concentrations tested.

in the presence of 50 μM alloxan than in its absence (Fig. 2). The level of DNA damage detected wit Endo III at 50 μM alloxan did not differ significantly from that in the absence of the drug. The repair enzymes we used recognized DNA modifications introduced by H_2O_2 (Endo III, Fpg) and MNNG (AlkA) in our experimental conditions (positive controls, results not shown). This suggests that alloxan might generate oxidized DNA bases with the

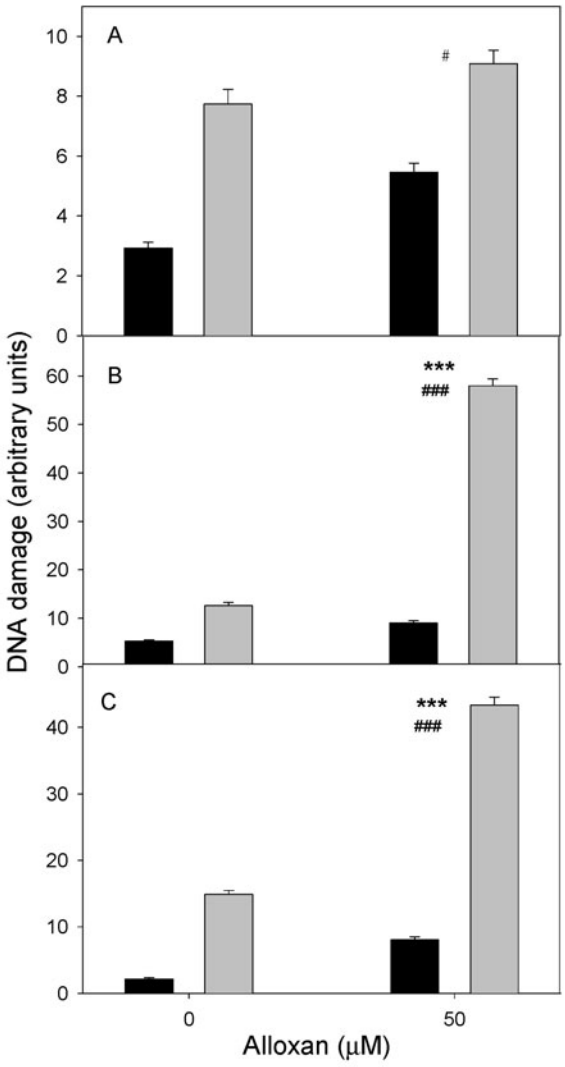

Figure 2. DNA damage measured by comet tail DNA in human lymphocytes exposed to alloxan for 1 h at 37°C with subsequent treatment (grey bars) with endonuclease III (A), formamidopyrimidine DNA-glycosylase (B) and 3-methyladenine-DNA glycosylase II (C) as compared with appropriate controls (black bars).

The figure shows mean results from three independent experiments. The number of cells analyzed in each treatment was 100; ***$P < 0.001$ for differences between enzyme treated and untreated groups in the presence and in the absence of alloxan; #$P < 0.05$, ###$P < 0.001$ as compared with the group treated with enzyme in the absence of alloxan (grey bars); error bars denote S.E.M.

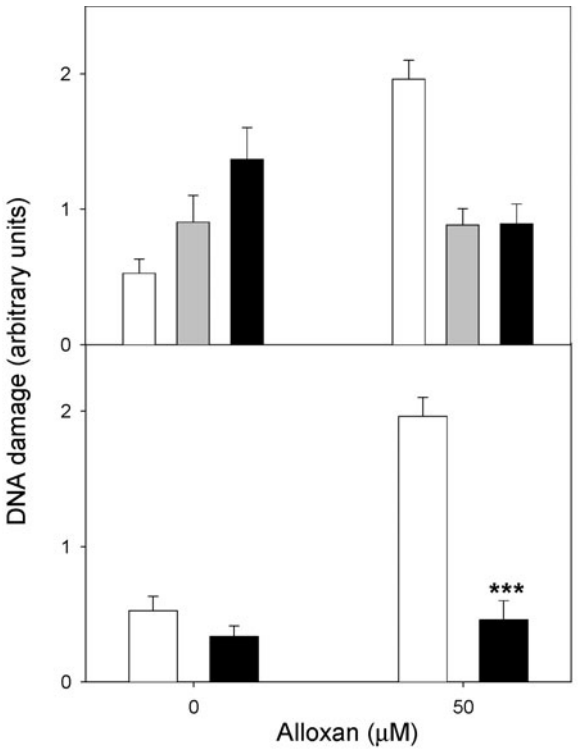

Figure 3. DNA damage measured by comet tail DNA in human lymphocytes exposed to alloxan for 1 h at 37°C in the presence of 10 (grey bars) or 50 μM (black bars) ebselen (2-phenyl-1,2-benzisoselenazol-3(2*H*)-one) (A) or the nitrone spin trap α-(4-pyridil-1-oxide)-*N*-t-butylnitrone (POBN) at 100 μM (black bars, B) as compared with appropriate controls (white bars).

The figure shows mean results from three independent experiments. The number of cells analyzed in each treatment was 100; **$P < 0.01$, ***$P < 0.001$; error bars denote S.E.M.

preference for purines and contribute to their alkylation. However, we must interpret the data obtained from repair enzymes analysis with special caution due to their broad substrate specificity. The obtained results can only point to the direction of further research to explore the mechanisms by which alloxan can modify the DNA bases in normal human lymphocytes. Ebselen and the spin trap POBN significantly decreased the level of DNA damage in the lymphocytes exposed to alloxan at 50 μM, although ebselen introduced significant ($P < 0.05$) damage to DNA in the absence of alloxan (Fig. 3). At present we are unable to give a rational explanation of the mechanism underlying the observed interaction of 50 μM

ebselen with alloxan. Preincubation of the cells with vitamin C or vitamin E at 10 or 50 μM also caused a decrease in the level of DNA damage (Fig. 4).

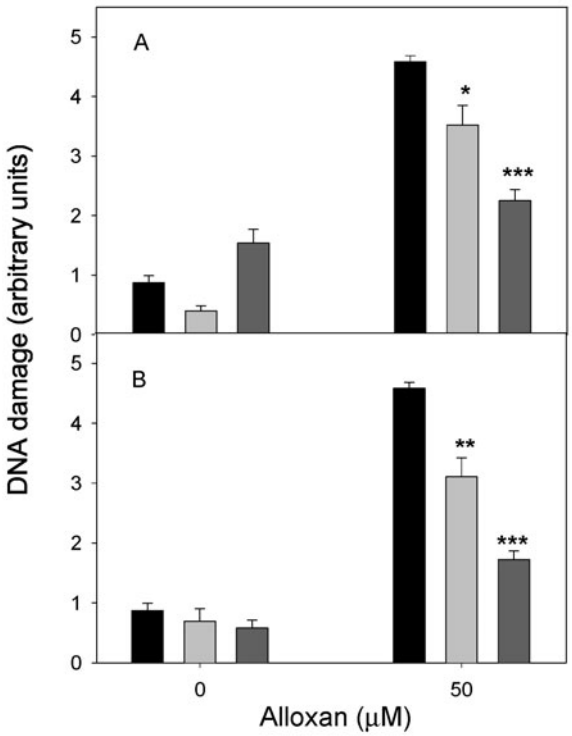

Figure 4. Figure 4. DNA damage measured by the comet tail DNA in human lymphocytes exposed for 1 h at 37°C to alloxan in the presence of 10 (light gray bars) or 50 μM (dark gray bars) vitamin C (A) or vitamin E (B) as compared with appropriate controls (black bars).

The figure shows mean results from three independent experiments. The number of cells analyzed in each treatment was 100; *$P < 0.05$, **$P < 0.01$; ***$P < 0.001$ error bars denote S.E.M.

The main aim of our study was to evaluate the free radical-based genotoxic potential of alloxan and the ability of some free radical scavengers to modulate the DNA-damaging effects of the drug. We clearly showed that alloxan can damage DNA in normal human lymphocytes, operating therefore as a genotoxic compound. The observed DNA damage might be due to the induction of DNA strand breaks and/or the formation of alkali labile sites, which can be transformed into strand breaks in the alkaline comet assay. Although the ability of alloxan to generate free radicals in the presence of suitable reducing agents, like reduced glutathione, and oxygen are well known (Bromme *et al.,* 2000), we have shown that it can induce DNA damage in normal cells. At present we are not able to elucidate whether or not these changes may be associated with diabetes. We also showed that various free radical scavengers may inhibit the DNA-damaging action of alloxan. This seems important for a better understanding of the role of this drug in generating pancreatic β-cells damage leading ultimately to diabetes mellitus. The deleterious action of alloxan is time- and dose-dependent, which reflects, to a certain extent, the balance between the antioxidant resources of the cells and the toxic potential of the drug. Altogether, our results support the theory that alloxan exerts its DNA-damaging action, at least in part, by the production of free radicals and that this action can be modulated by common antioxidants, which can easily supplement the diet.

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