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Life Sciences 76 (2004) 47–56

Life Sciences

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AZT induces oxidative damage to cardiac mitochondria: Protective effect of vitamins C and E

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Received 15 January 2004; accepted 14 June 2004

Abstract

AZT (zidovudine) is a potent inhibitor of HIV replication and a major antiretroviral drug used for AIDS treatment. A major limitation in the use of AZT is the occurrence of severe side effects. The aim of this work was to test whether AZT causes oxidative damage to heart mitochondria and whether this can be prevented by supranutritional doses of antioxidant vitamins. An experimental animal model was used in which mice were treated with AZT for 35 days (10 mg/kg/day) in drinking water. Animals treated with antioxidant vitamins were fed the same diet as controls but supplemented with vitamins C (ascorbic acid, 10 g/ kg diet) and E (α-dl-tocopherol, 0.6 g/kg diet) for 65 days before sacrifice. This resulted in a daily intake of 1250 mg/kg/day (vitamin C) and 75 mg/kg/day (vitamin E). Cardiac mitochondrial DNA (mtDNA) of mice treated with AZT had over 120% more oxo-dG (8-oxo-7,8-dihydro-2′ -deoxyguanosine, which is a biomarker of oxidative damage to DNA) in their mitochondrial DNA than untreated controls. AZT treatment also caused an increase in mitochondrial lipid peroxidation and an oxidation of mitochondrial glutathione. Dietary supplementation with supranutritional doses of the antioxidant vitamins C and E protected against these signs of mitochondrial oxidative stress. The oxidative effects of AZT are probably due to an increase in production of reactive oxygen species by mitochondria of AZT-

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0024-3205/$ - see front matter
doi:10.1016/j.lfs.2004.06.020

treated animals, raising the possibility that oxidative stress may play an important role in the cardiotoxicity of AZT.

Keywords: Zidovudine; mtDNA damage; Mitochondrial cardiomyopathy; Reactive oxygen species; Antioxidant vitamins

Introduction

AZT decreases human immunodeficiency virus replication and increases the number of CD_4+ cells. The incorporation of AZT monophosphate (AZT-MP) into viral DNA results in premature termination of DNA synthesis (Furman et al., 1990). The major limitation in the use of AZT is the occurrence of severe side effects (Yarchoan et al., 1989). The cardiac complications of AIDS include dilated cardiomyopathy which may be due to cardiotoxicity of AZT (Acierno, 1989; Coplan and Bruno, 1989). AZT induced mitochondrial myopathy has been attributed to depletion of mitochondrial DNA (Lewis et al., 1992). AZT is a mitochondrial toxin which affects the oxidation-phosphorylation coupling (Lampert et al., 1991) and disrupts the ultrastructure of cardiac mitochondria (Lewis et al., 1991). Cardiac mitochondrial DNA polymerase-γ from bovine heart is inhibited by phosphorylated zidovudine (AZT triphosphate) (Lewis et al., 1994). Szabados et al. (1999) have observed that AZT treatment increases the production of reactive oxygen species in the rat heart, raising the possibility that this may play an important role in the cardiotoxicity of AZT. Moreover, it has been recently reported an AZT-mediated inhibition of phosphate transport in rat heart mitochondria due to stimulation of superoxide anion mitochondrial production (Valenti et al., 2002).

Manifestations of mitochondrial diseases are thought to be due, at least in part, to an increased free radical formation (Luft, 1994; Johns, 1995). We found that there is a correlation between glutathione oxidation and mitochondrial DNA damage in aging (García de la Asunción et al., 1996). Both could be prevented by oral administration of antioxidant vitamins (García de la Asunción et al., 1996). We observed that AZT causes oxidative damage to skeletal muscle and liver mitochondria in mice, and found that reactive oxygen species-mediated processes can be an important factor in the development of myopathy and hepatopaty in zidovudine-treated AIDS patients (Viña et al., 1998; García de la Asunción et al., 1998; García de la Asunción et al., 1999).

The aim of the present study was to test whether AZT causes oxidative damage to cardiac mitochondria and whether this can be prevented by administration of supranutritional doses of the antioxidant vitamins C and E.

Materials and methods

Chemicals

RNAase (E.C.3.1.27.5) from bovine pancreas was purchased from Boehringer-Mannheim, (Germany), water and methanol (HPLC grade) from Merck (Germany), Nuclease P1 (E.C.3.1.30.1) from P. Citricum, alkaline phosphatase Type III-L (C.E.3.1.3.1) from E. Coli, glutathione-S-

transferase (E.C.2.5.1.18) from equine liver, reduced glutathione (GSH), oxidized glutathione (GSSG), 8-oxo-7,8-dihydro-2′ deoxyguanosine (8-oxo-dG), 3′ -azido-2′ ,3′ -dideoxythymidine (AZT), N-ethylmalemide (NEM), bathophenanthroline disulfonic acid (BPDS), 3-(N-morpholino) propane-sulfonic acid (MOPS), and all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals

Male OF1 mice (from IFFA-Credo, Barcelona, Spain) were used. They were maintained on a 12 h/12 h light/dark cycle at 22 °C. Mice were fed on a standard laboratory diet from Letica (Barcelona, Spain), containing: 590 g carbohydrates, 30 g lipids, and 160 g protein per kilogram of diet, and tap water ad libitum. Animals treated with antioxidant vitamins were fed the same diet but supplemented with vitamin C (ascorbic acid, 10 g/kg diet) and vitamin E (α-dl-tocopherol, 0.6 g/kg diet) for 65 days before sacrifice; this resulted in a daily intake of 1250 mg/kg body wt (vitamin C) and 75 mg/kg body wt (vitamin E). We have used the same high oral doses of antioxidant vitamins C and E as we previously did in other studies (García de la Asunción et al., 1998; García de la Asunción et al., 1999). These doses of vitamins C and E exhibited antioxidant properties in different mice tissues, such as skeletal muscle and liver, without any side effect. In addition, Lass et al. (1999) and Sumien et al. (2003) have treated mice orally with high doses of vitamin E (200 mg/Kg body wt/day), reporting increased α-tocopherol concentrations in plasma, whole heart and heart mitochondria after the treatment.

Treatment with vitamins was started one month before the treatment with AZT. We pretreated the animals for one month with vitamins C and E because previous work has shown that a minimum treatment period of 2–4 weeks is required to obtain maximum stable plasma and tissue levels of these vitamins (Levine et al., 1996; Kappus and Diplock, 1992). Animals were divided into three groups: (a) Controls, (b) mice treated with AZT, and (c) mice treated with AZT and dietary antioxidants. AZT was administered in drinking water (10 mg/kg body wt/day) for 35 days. When animals received an antioxidant-supplemented diet, their food intake and body weights were similar to those of the controls (results not shown). Five-month-old mice were anaesthetized with sodium pentobarbital (60 mg/kg body wt, i.p.) and killed by decapitation between 09:00 and 11:00 a.m. to minimize circadian variations of the parameters studied.

Mitochondrial isolation and preparation of mtDNA samples

Isolation of heart mitochondria was performed using differential centrifugation as described by Rickwood et al. (Rickwood et al., 1987). Extraction of mtDNA was accomplished according to the method described by La Torre et al. (La Torre et al., 1986) adapted to mammalian tissues (García de la Asunción et al., 1996). The purity of mtDNA was determined spectrophotometrically by measuring the absorbance ratio at 260/280 nanometers. The value found was 1.7–1.8, in agreement with previous studies (Richter et al., 1988).

The presence of mtDNA was confirmed by a restriction mapping as well as a PCR analysis using oligonucleotides specific for the mtDNA sequence (Richter et al., 1988). The amount of nuclear DNA contamination in our mtDNA preparations was less than 5%, as determined by gel electrophoresis followed by densitometry. The amount of DNA hydrolyzed for injection onto the HPLC-EC (high

performance liquid chromatography with electrochemical detection) was 30–100 μg as determined by UV analysis (absorbance at 260 nm). This was within the range suitable for analysis of oxo-dG in mitochondrial DNA samples (Beckman and Ames, 1996).

Measurement of 8-oxo-dG

This metabolite was measured as described by Shigenaga et al. (Shigenaga et al., 1994) based on reversed-phase HPLC-EC. Electrochemical detection of oxo-dG was performed using a dual coulometric detector (ESA Coulochem II, Model 5200, Bedford, USA) equipped with a 5011 analytical cell.

Measurement of reduced and oxidized glutathione

Reduced glutathione was measured spectrophotometrically using glutathione-S-transferase (Brigelius et al., 1983). Oxidized glutathione (GSSG) was assayed by an HPLC method with detection at 365 nm which we developed to measure GSSG in the presence of a large excess of GSH (Asensi et al., 1994; Viña et al., 1995).

Measurement of lipid peroxides

This was determined in cardiac mitochondria using an HPLC method described by Wong et al. (Wong et al., 1987) which determines malondialdehyde formed from lipoperoxides.

Mitochondrial lipoperoxides are hydrolyzed by boiling in diluted phosphoric acid. Malondialdehyde reacts with thiobarbituric acid to form an adduct which is detected by HPLC. The calibration curve was assayed using tetramethoxypropane which undergoes hydrolysis to liberate stoichiometric amounts of malondialdehyde (Gutteridge, 1975).

Measurement of production of reactive oxygen species by mitochondria

After isolation, cardiac mitochondria were suspended in ice-cold respiratory buffer (with 0.3 M sucrose, 1 mM EGTA, 5 mM MOPS, 0.1% BSA, 5 mM KH_2PO_4 adjusted to pH 7.4 with KOH) and were used to measure the production of reactive oxygen species (ROS) with an EPICS ELITE II flow cytometer (Coulter Electronics, Hialeah, Fl.). Dihydrorhodamine 123 was used as fluorochrome on incubation at 37 °C for 30 min. and was excited with an argon laser tuned at 488 nm. Colorless dihydrorhodamine 123 is oxidized outside mitochondria by H_2O_2-dependent reactions to the fluorescent product rhodamine 123 which stains mitochondria (Chen, 1988; Royall and Ischiropoulos, 1993). The gate was set on a logarithmic scale of forward and side scatter to always include mitochondria with the same morphological properties. Thus, changes in fluorescence were independent of the particle size.

Statistical analysis

Results are expressed as mean ± SD. Statistical analysis was performed by the least-significant difference test which consists of two steps: First an analysis of variance was performed. The null

hypothesis was accepted for all numbers of those set in which F was non-significant at the level of $P < 0.05$. Then, the sets of data in which F was significant were examined by the t-test using $p < 0.05$ as the critical limit.

Results

AZT causes oxidative damage to mitochondrial DNA

Fig. 1 shows that AZT causes an oxidation of heart mtDNA as shown by an increase in the levels of oxo-dG. Mitochondria from heart of control mice had a level of 8oxodG of 0.523 ± 0.042 pmol/μg mtDNA (n = 3). When mice were treated with AZT, oxo-dG levels in mtDNA were significantly higher ($p < 0.005$) than controls, i.e. 1.195 ± 0.184 pmol/μg mtDNA, (n = 3). The increase in oxo-dG was prevented by dietary administration of vitamins C and E: the level of oxo-dG found in this case in heart mtDNA was 0.554 ± 0.205 pmol/μg mtDNA (n = 3).

AZT causes mitochondrial lipid peroxidation

Malondialdehyde (MDA) levels are indicators of lipid peroxidation. Fig. 2 shows that MDA levels are significantly elevated in cardiac mitochondria of mice treated with AZT: MDA levels in control mice were 14.8 ± 0.5 nmols/mg protein (n = 3) and in AZT-treated mice they were 22.1 ± 4.7 nmol/mg protein (n = 4), $p < 0.05$.

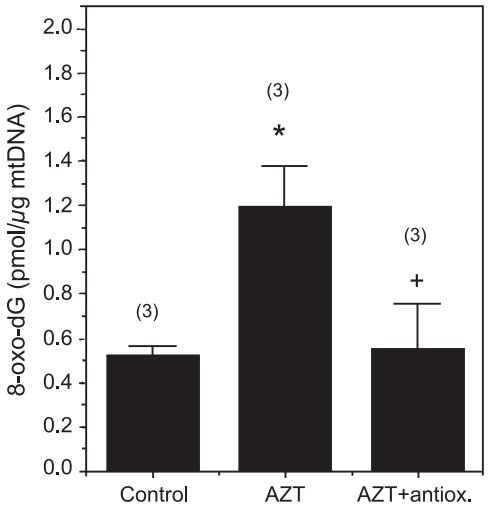

Fig. 1. Oxidative damage to mitochondrial DNA in mouse cardiac muscle from mice treated with AZT or AZT plus antioxidant vitamins. Data are mean ± SD. Statistical significance is expressed as follows: * Difference between AZT treated group versus untreated control group was significant at the level $P < 0.005$; and + difference between AZT plus antioxidant vitamin-treated group versus AZT-treated group was significant at the level $P < 0.05$.

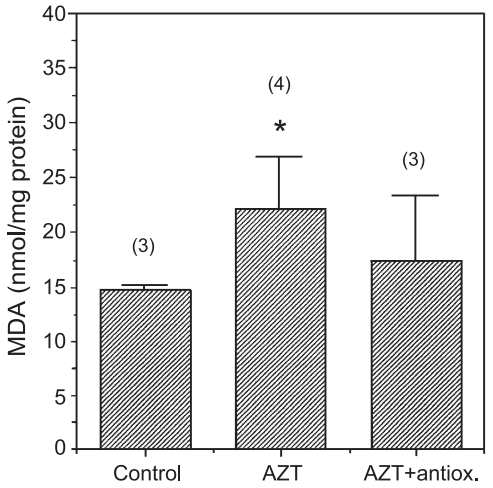

Fig. 2. Malondialdehyde levels in isolated mitochondria from mouse cardiac muscle treated with AZT or AZT plus antioxidant vitamins. Data are mean ± SD. Statistical significance is expressed as follows: * Difference between AZT treated group versus untreated control group was significant at the level $P < 0.005$; and + difference between AZT plus antioxidant vitamin-treated group versus AZT-treated group was significant at the level $P < 0.05$.

AZT causes an oxidation of mitochondrial glutathione

Table 1 shows that GSSG levels are increased in heart mitochondria from mice treated with AZT. Values of these mice are over 100% higher than those of controls. This was completely prevented by oral administration of antioxidant vitamins. The changes in GSSG resulted in parallel changes in the GSSG/GSH ratio (see Table 1).

AZT causes an increase in generation of reactive oxygen species by cardiac mitochondria

To investigate the possible cause of oxidative damage in cardiac mitochondria, we measured the effect of AZT on the rate of production of reactive oxygen species (ROS) in mitochondria isolated from heart. AZT caused a marked increase in ROS production by mitochondria. This was completely abolished when AZT-treated mice were fed a diet supplemented with vitamins C and E (see Fig. 3). However, we did not observe changes in mitochondrial membrane potential or particle size (results not shown).

Table 1
Levels of reduced glutathione (GSH), oxidized glutathione (GSSG) and GSSG/GSH ratio in isolated mitochondria from mouse cardiac muscle treated with AZT or AZT plus antioxidant vitamins

	Control	AZT	AZT+antiox.
GSH (nmol/mg prot.)	3.54 ± 1.10 (3)	4.63 ± 1.54(4)	5.04 ± 1.21(3)
GSSG (nmol/mg prot.)	0.051 ± 0.023 (4)	0.103 ± 0.059 (4)*	0.057 ± 0.015 (3)
GSSG/GSH (×100)	1.49 ± 0.89 (4)	3.58 ± 1.49 (4)*	1.49 ± 0.36 (4)+

Data are mean ± SD. Statistical significance is expressed as follows: *Difference between AZT treated group versus untreated control group was significant at the level $P < 0.05$; + difference between AZT treated group versus untreated control group was significant at the level $P < 0.05$.

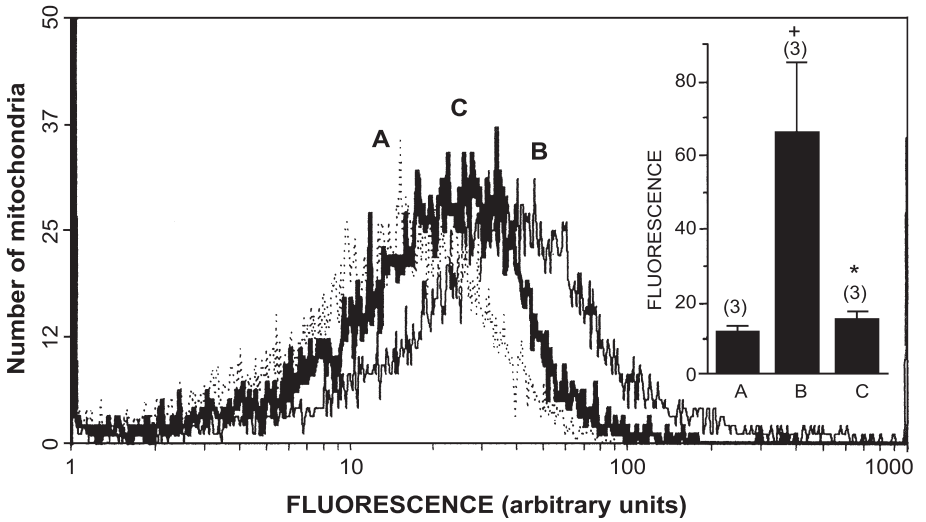

Fig. 3. Peroxide generation (expressed as arbitrary units of fluorescence) by isolated heart mitochondria from mice treated with AZT and antioxidant vitamins. A histogram of one representative experiment is shown, **A**: Control mice; **B**: Mice treated with AZT only; **C**: Mice treated with AZT and vitamins C plus E;. **Inset:** Values for three experiments are plotted. Peroxide formation in the AZT-treated group **(B)** was significatly higher ($p < 0.05$) than in groups **A** (control) or **C** (mice treated with AZT and antioxidant vitamins).

Therefore, changes in fluorescence were not due to alterations of the membrane potential and mitochondrial size and were only dependent on the rate of ROS production.

Discussion

AZT administration causes severe side effects, among them dilated cardiomyopathy (Acierno, 1989; Coplan and Bruno, 1989). This is probably due to mitochondrial alterations in heart muscle: mtDNA is depleted (Lewis et al., 1992), oxidation-phosphorylation coupling is altered (Lampert et al., 1991) and the fine ultrastructure of cardiac mitochondria is affected (Lewis et al., 1991). However, the mechanism of such cardiomyopathy is unknown. A recent report showed that reactive oxygen species (ROS) and peroxinitrites in mitochondria from AZT-treated rats may be the cause, at least in part, of the development of AZT-induced cardiomyopathy in rat (Szabados et al., 1999; Valenti et al., 2002).

We have studied the effect of AZT on mouse cardiac mitochondria and have found that AZT causes mitochondrial lipid peroxidation and oxidative damage to mitochondrial DNA (see Figs. 1 and 2). 8-oxo-dG is one of the best indicators of oxidative damage to DNA (Shigenaga et al., 1994). We previously found that changes in 8-oxo-dG correlate with other signs of oxidative stress such as glutathione oxidation (García de la Asunción et al., 1996). In accordance with this, other signs of oxidative stress, such as glutathione oxidation, also occur in mitochondria isolated from heart of AZT-treated animals (see Table 1). These effects are probably due to an increased peroxide formation by mitochondria from heart muscle of AZT-treated animals. The increase in ROS production may explain the signs of oxidative damage to mitochondrial macromolecules such as DNA, and the

oxidation of glutathione (Viña et al., 1998; García de la Asunción et al., 1998; García de la Asunción et al., 1999). Several studies have shown that AZT induces an inhibition of DNA polymerase gamma, responsible for mtDNA replication (Bolden et al., 1977). A possible link between this decrease in DNA replication and the increase in free radical production that we found is this: as a result of lowered mtDNA replication, mitochondrial turnover is decreased. Thus, mitochondria will not be properly renewed in the cell and aged mitochondria will remain there. We found that mitochondria from old animals produce more ROS than those from young ones and show signs of oxidative stress (Sastre et al., 1996). The fact that peroxide production, oxidation of mitochondrial DNA and oxidation of mitochondrial glutathione is prevented by oral administration of antioxidant vitamins C and E, provides a rationale for the use of these vitamins in the prevention of AZT-induced cardiotoxicity.

Conclusion

Cardiac mitochondrial DNA of mice treated with AZT exhibited over 120% more 8-oxo-dG (oxidized deoxyguanosine, a biomarker of oxidative damage to DNA) than untreated controls. AZT treatment also caused an increase in mitochondrial lipid peroxidation and oxidation of mitochondrial glutathione. Dietary supplementation with supranutritional doses of the antioxidant vitamins (C and E) protected against these signs of mitochondrial oxidative stress. An increased production of reactive oxygen species by mitochondria of AZT-treated animals appears to be responsible for the oxidative effects of AZT. Hence, oxidative stress may play an important role in the cardiotoxicity of AZT.

Acknowledgements

We thank Juana Belloch, Nuria García and Salvador Souan for their skillful technical assistance and Marilyn R. Noyes for revising the typescript. This work was supported by grants FIS 98/1462 and RCMN C03/08 from Instituto de Salud Carlos III to J.V and SAF 2002-00885 to FVP.

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