



ELSEVIER

Biochimica et Biophysica Acta 1411 (1999) 201–205

BIOCHIMICA ET BIOPHYSICA ACTA

BBA

Rapid report

Cardioselective and cumulative oxidation of mitochondrial DNA following subchronic doxorubicin administration¹J. Serrano ^a, C.M. Palmeira ^b, D.W. Kuehl ^a, K.B. Wallace ^{c,*}^a NHEERL, Midcontinent Ecology Division, U.S. EPA, Duluth, MN, USA^b Centro de Neurociencias, Department of Zoology, Universidade de Coimbra, 3000 Coimbra, Portugal^c Department of Biochemistry and Molecular Biology, University of Minnesota School of Medicine, Duluth, MN, USA

Received 28 December 1998; accepted 11 January 1999

Abstract

We recently reported the preferential accumulation of 8-hydroxydeoxyguanosine (8OHdG) adducts in cardiac mitochondrial DNA (mtDNA) following acute intoxication of rats with doxorubicin (C.M. Palmeira et al., *Biochim. Biophys. Acta*, 1321 (1997) 101–106). The concentration of 8OHdG adducts decreased to control values within 2 weeks. Since conventional antineoplastic therapy entails repeated administration of small doses of doxorubicin, it was of interest to characterize the kinetics for the accumulation and repair of 8OHdG adducts in the various DNA fractions. Weekly injections of doxorubicin (2 mg/kg, i.p.) to adult male Sprague–Dawley rats caused a cumulative dose-dependent increase in the concentration of 8OHdG adducts in both mtDNA and nuclear DNA (nDNA) from heart and liver. Following six weekly injections, the concentration of 8OHdG in cardiac mtDNA was 50% higher than liver mtDNA and twice that of cardiac nDNA. In contrast to the rapid repair of 8OHdG observed during the first days following an acute intoxicating dose of doxorubicin, the concentration of 8OHdG adducts remained constant between 1 and 5 weeks following the last injection. This was true for all DNA fractions examined. The cardioselective accumulation and persistence of 8OHdG adducts to mtDNA is consistent with the implication of mitochondrial dysfunction in the cumulative and irreversible cardiotoxicity observed clinically in patients receiving doxorubicin cancer chemotherapy. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Adriamycin; Doxorubicin; Mitochondrial DNA; 8-Hydroxydeoxyguanosine

8-Hydroxydeoxyguanosine (8OHdG) is one of several DNA adducts and deletions that accumulate over the life span of an individual [2–7]. Formation of 8OHdG adducts is believed to reflect the age-dependent progressive uncoupling of oxidative metabolism and generation of free radical species of oxygen, including the hydroxyl free radical which hydroxylates deoxyguanosine preferentially at C₈ [8]. As such, 8OHdG is suggested to provide a reliable indicator of the metabolic history and bioenergetic capacity of the organism.

* Corresponding author. Fax: +1-218-726-8014;

E-mail: kwallace@d.umn.edu

¹ Supported in part by NIH Grant HL58016. The manuscript was not subjected to internal Agency review and, thus, the opinions are those of the authors and are not officially endorsed by the U.S. Environmental Protection Agency. Mention of trade names or commercial products does not constitute endorsement nor is it intended to imply any form of judgment by the authors.

8OHdG adducts accumulate to varying proportions in different tissues, being most prevalent in mitotically fixed, metabolically active tissues such as muscle and brain [9]. Furthermore, these adducts accumulate to a larger extent in mitochondrial DNA (mtDNA) than in nuclear DNA (nDNA). This preferential accumulation of adducts may reflect either more rapid rates of generation of oxygen free radicals or slower rates of repair of the genetic damage in the mitochondrion. For example, the age-dependent increase in 8OHdG adducts to mtDNA correlates with the lower coupling efficiency and greater free radical generation from the respiratory chain of aged animals [6,7,10,11]. It is also consistent with the initial reports of deficient mitochondrial DNA repair activities compared to that associated with the nucleus [12–18]. Regardless, it is the accumulation of these adducts that is proposed to account for the progressive decline in cognitive and physical performance that accompanies aging.

The accumulation of 8OHdG adducts in mtDNA is not limited to the normal physiological aging process. Accelerated rates of accumulation of these adducts may also account for the pre-senescent cognitive and myopathic changes associated with numerous degenerative disorders, including; Alzheimer's disease, progressive external ophthalmoplegia, Kearns–Sayre syndrome, MELAS, and in Parkinson's disease [19–21]. Furthermore, the accumulation of these adducts is also influenced by extrinsic factors such as chemical exposures. Substantial increases in 8OHdG adducts to nuclear DNA occurs in response to chronic feeding of rats with ethanol [22] or to drugs that cause the proliferation of peroxisomes in liver tissue [23]. The tissue-selective increase of 8OHdG adducts to hepatic, but not renal, DNA following short-term intoxication of rats with perfluorooctanoic acid or perfluorodecanoic acid correlates with the target-organ specificity of these chemicals, inferring a cause-and-effect relationship [23].

In view of the relationship between mtDNA transcription and bioenergetic capacity of a tissue, it is feasible that the accumulation of mitochondrial mutations and the accompanying interference with template activity, may be responsible for many of the metabolic and bioenergetic deficits associated not only with aging and neurodegenerative disease, but

also with chemical intoxications. We recently reported that a single acute injection of rats with doxorubicin (15 mg/kg, i.p.) causes the preferential accumulation of 8OHdG adducts to DNA in heart compared to liver and that in both tissues, the concentration of adducts was 2-fold greater in mtDNA than nDNA [1]. Of particular interest was the fact that the increase in 8OHdG was transient in all tissue fractions, returning to near control values within one to two weeks. From this, we concluded that doxorubicin is a mitochondrial mutagen, particularly in heart, and that any resulting interference with mtDNA transcription may account for the bioenergetic failure associated with doxorubicin cardiomyopathy. However, doxorubicin is typically administered subchronically, in divided doses over a period of several months. The question then becomes whether the kinetics of adduct formation and repair or replacement are sufficient to warrant a concern for the potential summation of genetic damage following repetitive dosing with the drug.

To address the issue of cumulative toxicity, adult male Sprague–Dawley rats (Harlan Labs, Madison, WI) received 2–8 weekly i.p. injections of either doxorubicin (2 mg/kg) or an equivalent volume of saline (1 ml/kg). The animals were housed in AAALAC-accredited, climate-controlled facilities and allowed free access to food (Purina rodent diet no. 5001) and water. Animals were killed by decapitation 1–5 weeks after the last injection and the heart and liver immediately excised to cold isotonic buffer. The tissues were homogenized in a mixture containing 225 mM mannitol, 75 mM sucrose, 1 mM EGTA and 10 mM MOPS (pH 7.4), and the mitochondrial and nuclear fractions isolated by differential centrifugation [1]. Both fractions were then solubilized in 2.7% SDS and digested with proteinase K (400 µg/ml) for 3 h at 37°C. DNA from the respective fraction was extracted into phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with isopropanol at room temperature. The precipitate was digested with RNases A and T1 and the DNA re-precipitated and hydrolyzed to the individual nucleic acids by digestion with DNase I, nuclease P1, and alkaline phosphatase. The nucleic acids were stable for up to 5 days when stored frozen at pH 7.8. The concentration of 8OHdG in both the mtDNA and nDNA fractions of heart and liver were quantified by LC/

ESI/MS/MS [1,24] and is expressed relative to the concentration of unmodified deoxyguanosine (dG) bases.

The concentration of 8OHdG adducts to mtDNA and nDNA of both heart and liver of saline-injected rats was less than 5 adducts/ 10^5 dG and did not change over the 11-week course of the experiment. In contrast, 8OHdG ranged between 10 and 25 adducts/ 10^5 dG after as few as two weekly injections of doxorubicin (Fig. 1). 8OHdG accumulated in cardiac nDNA in proportion to the total cumulative dose of doxorubicin, the concentration increasing in near linear fashion from 17 adducts/ 10^5 dG after the second injection to 38/ 10^5 dG after eight weekly injections. This contrasts with liver nDNA where the concen-

tration of 8OHdG reached a maximum of approximately 27/ 10^5 dG after the fourth dose. Additional injections of doxorubicin did not cause further increases in 8OHdG adducts to liver nDNA, suggesting that a steady state was reached wherein the rate of adduct formation equaled or was exceeded by the rate of repair or replacement through cell turnover.

8OHdG adducts accumulated to a much greater extent (ca. 2-fold) in mtDNA than nDNA of both heart and liver, with the highest concentration of adducts occurring in cardiac mtDNA (60–65 8OHdG/ 10^5 dG 1 week after eight injections, which is about 50% higher than that for liver mtDNA). In both tissues, the concentration of adducts increased with successive doses of doxorubicin. However, the incremental increase in 8OHdG became less with successive doses, suggesting the eventual attainment of a steady state that is determined by the balance of the corresponding rates of formation and repair or replacement.

Interestingly, the concentration of 8OHdG adducts did not decrease over a 5-week period following discontinuation of drug administration in any of the tissue fractions examined. This contrasts with the rapid repair or dilution that occurs during the first week following a single high dose of doxorubicin [1]. The data indicate that essentially all of the repair or replacement through cell proliferation is complete within the first week. Adducts which are not repaired within this timeframe are stable and accumulate with successive weekly injections of doxorubicin.

The preferential accumulation of adducts in heart as compared to liver indicates either that the rate of formation of 8OHdG is greater or that the rate of repair is lower in cardiac tissue. Although there are no data for the formation of 8OHdG, the stimulation of superoxide anion radical generation by doxorubicin is 5-fold greater in heart compared to liver mitochondria [25]. Scheulen et al. [26,27] reported that the rate of activation and covalent binding of doxorubicin metabolites to microsomal proteins *in vitro* is almost 10-fold greater for heart than liver cell fractions. It is possible that this reflects in part the fact that cardiac tissue is mitotically fixed and cannot replace altered genomes and oxidized proteins by stimulating cell proliferation. Dusonchet et al. [28] found that the rate of unscheduled DNA synthesis following subchronic doxorubicin administration to

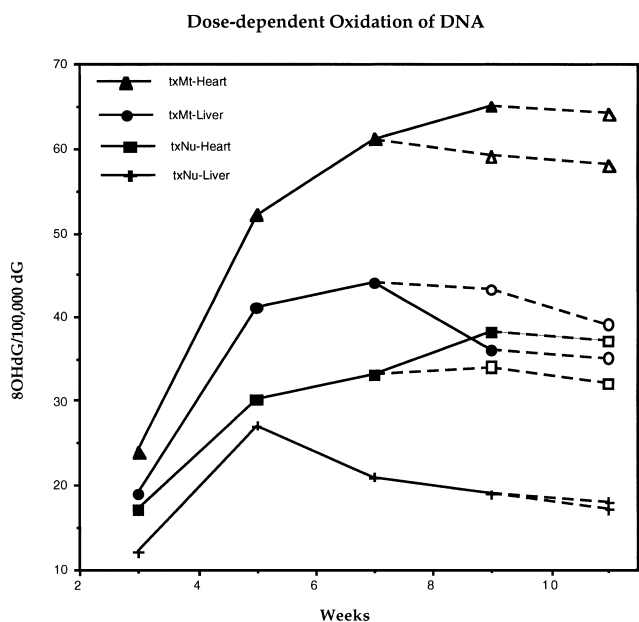


Fig. 1. Cumulative-dose-dependent oxidation of mitochondrial and nuclear DNA. Rats received from two to eight weekly injections of 2 mg (i.p.) doxorubicin/kg (tx). The concentration of 8OHdG was quantified by LC/ESI/MS/MS and expressed relative to the concentration of unmodified dG. Rats were killed 1 week following the last injection and both mtDNA and nDNA isolated from heart and liver (solid lines). Data points connected by broken lines represent the concentration of adducts in samples harvested from animals that received either six or eight weekly doses of doxorubicin then allowed to recover for up to 5 weeks with no further injections. Each point represents the mean of duplicate measurements from pooled tissues from two rats each. The corresponding concentration of adducts to cardiac and liver nDNA and mtDNA from paired control rats that received parallel injections of isotonic NaCl was invariably less than 5/ 10^5 dG at all treatment times.

mice is substantially lower in heart than liver. Thus, the cardioselective accumulation of 8OHdG is consistent with high rates of formation and a lower capacity for repair or replacement of oxidized DNA adducts in heart.

The preferential accumulation of mtDNA adducts agrees with previous reports [1,7] and suggests a higher rate of doxorubicin-stimulated hydroxyl radical generation and 8OHdG formation and/or a slower rate of repair in the mitochondrion compared to the nucleus. Although little is known regarding the relative rates of drug-induced free radical generation in the nuclear and mitochondrial compartments, the metabolism of doxorubicin by isolated intact rat liver nuclei is very low, amounting to only 5% that of the microsomal fraction of the same tissue [29]. In contrast, doxorubicin stimulates a very high rate of free radical generation from the mitochondrial electron transport chain [30,31]. Giulivi et al. [32] characterized a strong correlation between the rate of generation of hydroxyl free radicals in submitochondrial particles and the occurrence of 8OHdG adducts to the mitochondrial genome. Accordingly, it is probable that doxorubicin stimulates high rates of formation of 8OHdG in the immediate vicinity of the mtDNA. Once formed, however, the rates of repair of mtDNA adducts may be no different than that which occurs in the nucleus [14,30], despite initial reports of deficiencies in mtDNA repair enzymes. Thus, the preferential accumulation of mtDNA adducts more likely reflects a more rapid rate of formation than a deficiency in DNA repair.

In summary, cumulative doses of doxorubicin cause a progressive accumulation of 8OHdG adducts to both nDNA and mtDNA that are stable for up to 5 weeks following discontinuation of drug dosing. Inasmuch as these adducts accumulate at doses that do not alter body or organ weights, this appears to represent an early response to doxorubicin intoxication. The cardioselective accumulation of mitochondrial adducts is of particular interest in that it contrasts with the fact that the drug itself is retained much longer in the liver than heart and that it concentrates in the nuclear fraction (as opposed to mitochondrial fraction) in both tissues [33]. This disparity indicates that it is not the presence of the drug or its major metabolites that is the principal determinant of 8OHdG adduct accumulation in the different

tissue fractions. Since expression of the mitochondrial genome is essential to the integrity of the respiratory chain, the preferential oxidation of mtDNA would indicate the eventual disruption of mitochondrial structure and deterioration in bioenergetic function. Both phenomena are early and prominent features associated with the experimental manifestation of doxorubicin-induced cardiomyopathy [34–37]. Accordingly, one could invoke this dose-dependent accumulation of cardiac mtDNA adducts as a potential cause of the cumulative and irreversible cardiomyopathy observed clinically.

References

- [1] C.M. Palmeira et al., Preferential oxidation of cardiac mitochondrial DNA following acute intoxication with doxorubicin, *Biochim. Biophys. Acta* 1321, (2) (1997) 101–106.
- [2] B.N. Ames, Endogenous DNA damage as related to cancer and aging, *Mutat. Res.* 214, (1) (1989) 41–46.
- [3] B.N. Ames, Endogenous oxidative DNA damage, aging, and cancer, *Free Radic. Res. Commun.* 7, (3–6) (1989) 121–128.
- [4] M. Hayakawa et al., Age-associated accumulation of 8-hydroxydeoxyguanosine in mitochondrial DNA of human diaphragm, *Biochem. Biophys. Res. Commun.* 179, (2) (1991) 1023–1029.
- [5] K. Katsumata et al., Fragmentation of human heart mitochondrial DNA associated with premature aging, *Biochem. Biophys. Res. Commun.* 202, (1) (1994) 102–110.
- [6] P. Mecocci et al., Oxidative damage to mitochondrial DNA shows marked age-dependent increases in human brain, *Ann. Neurol.* 34, (4) (1993) 609–616.
- [7] C. Richter, Oxidative damage to mitochondrial DNA and its relationship to ageing, *Int. J. Biochem. Cell Biol.* 27, (7) (1995) 647–653.
- [8] M. Dizdaroglu, Chemical determination of free radical-induced damage to DNA, *Free Radic. Biol. Med.* 10, (3–4) (1991) 225–242.
- [9] N. Arnheim, G. Cortopassi, Deleterious mitochondrial DNA mutations accumulate in aging human tissues, *Mutat. Res.* 275, (3–6) (1992) 157–167.
- [10] M. Takasawa et al., Age-associated damage in mitochondrial function in rat hearts, *Exp. Gerontol.* 28, (3) (1993) 269–280.
- [11] M. Hayakawa et al., Age-associated oxygen damage and mutations in mitochondrial DNA in human hearts, *Biochem. Biophys. Res. Commun.* 189, (2) (1992) 979–985.
- [12] S. Ikeda, K. Ozaki, Action of mitochondrial endonuclease G on DNA damaged by L-ascorbic acid, peplomycin, and cis-diamminedichloroplatinum (II), *Biochem. Biophys. Res. Commun.* 235, (2) (1997) 291–294.
- [13] O.A. Olivero et al., Preferential formation and decreased removal of cisplatin-DNA adducts in Chinese hamster ovary

- cell mitochondrial DNA as compared to nuclear DNA, *Mutat. Res.* 391, (1–2) (1997) 79–86.
- [14] B.G. Taffe et al., Gene-specific nuclear and mitochondrial repair of formamidopyrimidine DNA glycosylase-sensitive sites in Chinese hamster ovary cells, *Mutat. Res.* 364, (3) (1996) 183–192.
- [15] A. Sancar, DNA repair in humans, *Annu. Rev. Genet.* 29 (1995) 69–105.
- [16] S.P. LeDoux et al., Repair of N-methylpurines in the mitochondrial DNA of xeroderma pigmentosum complementation group D cells, *Carcinogenesis* 14, (5) (1993) 913–917.
- [17] S.P. LeDoux et al., Repair of mitochondrial DNA after various types of DNA damage in Chinese hamster ovary cells, *Carcinogenesis* 13, (11) (1992) 1967–1973.
- [18] G. Singh, E. Maniccia-Bozzo, Evidence for lack of mitochondrial DNA repair following cis-dichlorodiammineplatinum treatment, *Cancer Chemother. Pharmacol.* 26, (2) (1990) 97–100.
- [19] P. Mecocci, U. MacGarvey, M.F. Beal, Oxidative damage to mitochondrial DNA is increased in Alzheimer's disease, *Ann. Neurol.* 36, (5) (1994) 747–751.
- [20] M. Tanaka et al., Accumulation of deletions and point mutations in mitochondrial genome in degenerative diseases, *Ann. NY Acad. Sci.* 786 (1996) 102–111.
- [21] T. Mitsui et al., Oxidative damage to skeletal muscle DNA from patients with mitochondrial encephalomyopathies, *J. Neurol. Sci.* 139, (1) (1996) 111–116.
- [22] A. Cahill, X. Wang, J.B. Hoek, Increased oxidative damage to mitochondrial DNA following chronic ethanol consumption, *Biochem. Biophys. Res. Commun.* 235, (2) (1997) 286–290.
- [23] P.J. Sausen et al., Elevated 8-hydroxydeoxyguanosine in hepatic DNA of rats following exposure to peroxisome proliferators: relationship to mitochondrial alterations, *Carcinogenesis* 16, (8) (1995) 1795–1801.
- [24] J. Serrano et al., Determination of 8-hydroxydeoxyguanosine in biological tissue by liquid chromatography/electrospray ionization-mass spectrometry/mass spectrometry, *Rapid Commun. Mass. Spectrom.* 10, (14) (1996) 1789–1791.
- [25] T. Adachi et al., Relation between cardiotoxic effect of adriamycin and superoxide anion radical, *J. Pharmacobiodyn.* 6, (2) (1983) 114–123.
- [26] M.E. Scheulen et al., Covalent protein binding of reactive adriamycin metabolites in rat liver and rat heart microsomes, *J. Cancer Res. Clin. Oncol.* 103, (1) (1982) 39–48.
- [27] M.E. Scheulen, H. Kappus, Metabolic activation of adriamycin by NADPH-cytochrome P-450 reductase, rat liver and heart microsomes and covalent protein binding of metabolites, *Adv. Exp. Med. Biol.* 136, (Pt. A) (1981) 471–485.
- [28] L. Dusonchet et al., The influence of multiple treatment with doxorubicin or 4'-deoxy-doxorubicin on the DNA synthesis and morphology of mouse heart, *Int. J. Tissue React.* 6, (1) (1984) 81–89.
- [29] K.B. Wallace, Aglycosylation and disposition of doxorubicin in isolated rat liver nuclei and microsomes, *Drug Metab. Dispos.* 14, (4) (1986) 399–404.
- [30] F.M. Yakes, B. Van Houten, Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress, *Proc. Natl. Acad. Sci. USA* 94, (2) (1997) 514–519.
- [31] R. Ogura et al., Electron spin resonance studies on the mechanism of adriamycin-induced heart mitochondrial damages, *Cancer Res.* 51, (13) (1991) 3555–3558.
- [32] C. Giulivi, A. Boveris, E. Cadenas, Hydroxyl radical generation during mitochondrial electron transfer and the formation of 8-hydroxydeoxyguanosine in mitochondrial DNA, *Arch. Biochem. Biophys.* 316, (2) (1995) 909–916.
- [33] B.J. Marafino Jr., S.N. Giri, D.M. Siegel, Pharmacokinetics, covalent binding and subcellular distribution of [3H]doxorubicin after intravenous administration in the mouse, *J. Pharmacol. Exp. Ther.* 216, (1) (1981) 55–61.
- [34] R.C. Aversano, P.J. Boor, Histochemical alterations of acute and chronic doxorubicin cardiotoxicity, *J. Mol. Cell Cardiol.* 15, (8) (1983) 543–553.
- [35] L.E. Solem, L.J. Heller, K.B. Wallace, Dose-dependent increase in sensitivity to calcium-induced mitochondrial dysfunction and cardiomyocyte cell injury by doxorubicin, *J. Mol. Cell Cardiol.* 28, (5) (1996) 1023–1032.
- [36] L.E. Solem, T.R. Henry, K.B. Wallace, Disruption of mitochondrial calcium homeostasis following chronic doxorubicin administration, *Toxicol. Appl. Pharmacol.* 129, (2) (1994) 214–222.
- [37] M. Praet, J.M. Ruyschaert, In-vivo and in-vitro mitochondrial membrane damages induced in mice by adriamycin and derivatives, *Biochim. Biophys. Acta* 1149, (1) (1993) 79–85.