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Preferential oxidation of cardiac mitochondrial DNA following acute intoxication with doxorubicin

C.M. Palmeira^a, J. Serrano^b, D.W. Kuehl^b, K.B. Wallace^{a,*}^a Department of Biochemistry and Molecular Biology, University of Minnesota, School of Medicine, 10 University Drive, Duluth, MN 55812, USA^b Midcontinent Ecology Division, U.S. EPA, Duluth, MN, USA

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Abstract

The purpose of this investigation was to determine whether acute doxorubicin intoxication causes a preferential accumulation of 8-hydroxydeoxyguanosine (8OHdG) adducts to mitochondrial DNA (mtDNA) as opposed to nuclear DNA (nDNA), particularly in cardiac tissue. Adult male rats received a single i.p. bolus of doxorubicin (15 mg/kg) and were killed 1–14 days later. Acute intoxication with doxorubicin caused a 2-fold greater increase in 8OHdG adducts to mtDNA compared to nDNA, the concentration of adducts to both nDNA and mtDNA being 20%–40% greater for heart as opposed to liver. For both tissues, the relative abundance of adducts was highest at the earliest time-point examined (24 h) and decreased to control values by 2 weeks. The temporal dilution of 8OHdG adducts was not the result of cell hyperplasia and was only partially due to amplification of the mitochondrial genome, most probably via an increase in DNA copy number rather than a stimulation of mitochondrial biogenesis. © 1997 Elsevier Science B.V.

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8-Hydroxydeoxyguanosine (8OHdG) adducts of both nuclear and mitochondrial DNA (nDNA and mtDNA, respectively) accumulate over the life span of an individual [1–5]. The extent of accumulation varies among tissues, being most pronounced in mitotically fixed, aerobically active tissues such as brain and cardiac and skeletal muscle [6]. The origin of these adducts is believed to be the reaction of free radical species of oxygen, specifically the hydroxyl free radical, with DNA to cause the preferential hydroxylation of deoxyguanosine at C₈ [7]. In mito-

chondria, which are deficient in DNA repair enzymes [8–11], the 8OHdG adducts accumulate with repeated oxidative insult. Contrast this with nuclei which have a proficient DNA repair capacity.

The accumulation of 8OHdG adducts of mtDNA is dependent not only on intrinsic metabolic rates, but it is also influenced by extrinsic factors such as environmental exposure histories. For example, exposure of animal cells in culture to polyaromatic hydrocarbons, nitrosamines or azo dyes results in a 40–500-fold increase in concentration of mtDNA adducts compared to nDNA adducts [12–15]. A similar preferential accumulation of mtDNA adducts occurs following in vivo exposure to carbon tetrachloride, nitrosamines or aflatoxin [10,15–18]. The ratio of

* Corresponding author. Fax: (1) (218) 726-8014; E-mail: kwallace@d.umn.edu

mtDNA/nDNA adducts ranges between 2 and 50, depending on the toxic agent, the tissue, and the time elapsed since exposure.

The accumulation of mtDNA adducts and the associated interference with genomic template activity may explain many of the metabolic and bioenergetic deficits associated with chemical intoxication. For example, acute intoxication of rats with perfluorooctane sulfonamide results in a preferential accumulation of 8OHdG adducts of mtDNA in liver but not kidney tissue [19]. This may reflect tissue-specific sites of metabolic activation and correlates with the fact that perfluorooctane sulfonamide causes peroxisome proliferation and hepatocellular carcinomas but is not suspected of being nephrotoxic [20]. Accordingly, analysis of 8OHdG mtDNA adducts may provide a reliable biomarker for tissue-specific oxidative injury in response to oxidant exposure *in vivo*.

One such agent that is of considerable clinical importance is the anthracycline antibiotic, doxorubicin (Adriamycin®), which is a potent, broad-spectrum antineoplastic agent used for treating a number of both solid tumors and leukemias. However, the clinical utility is limited by the incidence of a life-threatening cardiomyopathy in patients receiving a cumulative dose exceeding 550 mg/m² [21]. The mechanism of cardiotoxicity involves redox cycling of the drug on the endoplasmic reticulum and/or mitochondrial membranes to liberate highly reactive free radical species of oxygen [22]. In cardiac tissue, in which mitochondria comprise up to 50% of the total cell volume, the principal intracellular site of redox cycling is on complex I of the respiratory chain [23,24]. We recently reported an 8-fold increase in concentration of 8OHdG adducts of nDNA in rat liver following acute intoxication with doxorubicin [25]. Cho et al. [26] demonstrated an organ-specific accumulation of 8OHdG adducts to nDNA in kidney, as opposed to liver, following acute intoxication with doxorubicin. This is consistent with the drug causing kidney tumors but not liver tumors. The purpose of the present investigation was to determine if there is a preferential accumulation of 8OHdG adducts of mtDNA compared to nDNA following acute doxorubicin exposure and whether there is any selectivity for heart tissue.

Male Sprague–Dawley rats (200–250 g) were purchased from Harlan Sprague–Dawley (Madison, WI)

and housed in an AAALAC-accredited, climate-controlled (21°C, 14/10 h light cycle) animal care facility. The animals were allowed to acclimate for at least 5 days with free access to food (Purina Rodent Diet No. 5001) and water. Five rats each were injected *i.p.* with a single bolus of either 15 mg/kg doxorubicin (Adriamycin®, Adria Laboratories, Columbus, OH) or an equivalent volume (1 ml/kg) of 0.9% NaCl. The rats were later killed by decapitation and both the heart and liver quickly excised to cold (4°C) MSEM (225 mM mannitol/75 mM sucrose/1 mM EGTA/10 mM MOPS, pH 7.4).

The isolation of both heart and liver mitochondria was precisely as described previously [27,28]. To 1.5 g of heart tissue was added 2.5 ml of protease Type VIII (Sigma Chemical, St. Louis, MO) at 5 u/ml in MSEM. The tissue was homogenized gently for 30 s (glass-Teflon Potter–Elvehjem) then immediately diluted with 26.25 ml fresh, protease-free MSEM (4°C) and the homogenization continued for an additional 60 s. The homogenate was centrifuged at 8000 × *g* for 10 min at 4°C. The pellet was resuspended in 30 ml fresh MSEM and stored on ice.

The liver was homogenized in 20 vols. (ml/g) protease-free MSEM. Nuclei were separated from mitochondria by centrifuging both the liver homogenate and the resuspended pellet of the heart homogenate at 1500 × *g* for 10 min at 4°C. The supernatants containing the mitochondria were separated and stored on ice. The pellets were washed by resuspending in fresh MSEM (20 vols.). This was diluted 6-fold with additional MSEM then re-centrifuged at 1500 × *g* for 10 min at 4°C. The washed nuclear pellet was resuspended in 4 vols. of TSE (100 mM Tris/160 mM sucrose/10 mM EDTA, pH 7.5) and stored on ice.

Mitochondria were recovered from the original 1500 × *g* supernatants by centrifuging for 15 min at 12 000 × *g*. The mitochondrial pellets were resuspended in 20 vols. fresh MSEM and re-centrifuged at 12 000 × *g* for 15 min. The final mitochondrial pellets were resuspended in 2 vols. (ml/g original tissue) of 100 mM Tris/160 mM sucrose (pH 7.5) to which was added 2.5 μg/ml DNase I (Sigma, Type IV). Mitochondrial cytochrome oxidase and citrate synthase activities were measured prior to digesting with DNase. The remaining sample was digested for 30 min at 37°C and the reaction stopped by adding

EDTA to a final concentration of 25 mM. The mitochondria were recovered by centrifugation ($11\,000 \times g$ for 15 min) and re-suspended in 2 vols. of fresh TSE.

Both the nuclear and mitochondrial fractions were then solubilized in 2.7% SDS and digested with proteinase K (400 $\mu\text{g}/\text{ml}$, Sigma) for 3 h at 37°C . DNA was extracted with 2 vols. of phenol/chloroform/iso-amyl alcohol (25:24:1). The phases were separated by centrifugation at $1000 \times g$ for 5 min. The aqueous phase was recovered by aspiration and re-extracted. Nucleic acids were precipitated by adding 0.1 vols. of 3 M potassium acetate and 2 vols. cold ethanol, then left at -20°C overnight. The DNA precipitate was collected by centrifugation ($20\,000 \times g$ for 2 h at 4°C) and resuspended in 400 μl 100 mM Tris/10 mM EDTA (pH 7.5). Contaminating RNA was removed by digesting for 2 h at 37°C with RNases A and T_1 (ca. 2 and 125 units, respectively). The digested DNA was precipitated in potassium acetate/ethanol overnight at -20°C . The DNA precipitate was collected by centrifugation (20 min at maximum speed in a microfuge) and washed once with cold 70% ethanol.

The washed DNA was then hydrolyzed by sequential digestion with DNase I, nuclease P1, and alkaline phosphatase and the final deoxynucleotides stored frozen at pH 7.8 until analyzed [29]. The concentration of 8OHdG adducts to each of the corresponding DNA fractions was quantified by LC/ESI/MS/MS (liquid chromatography/electrospray ionization/tandem mass spectrometry) as previously described [25]. 8OHdG standards were synthesized by oxidizing commercial deoxyguanosine (dG; Sigma Chemical) in a Fenton reaction medium consisting of 1 mM hydroquinone, 5 mM H_2O_2 , 0.1 mM FeCl_3 , and 0.5 mM EDTA [30]. The instrument was calibrated with these synthetic standards on the basis of the MS/MS peak area response. The data are expressed as the relative abundance of 8OHdG adducts per 100 000 dG nucleosides.

Male rats injected with saline gained approximately 5.3 g/day, averaged over 14 days. Doxorubicin-intoxicated rats, however, failed to gain weight (data not shown). Although the increase in liver mass was similar for the two groups, there was no increase in heart weight for animals treated acutely with doxorubicin (data not shown). In control animals, heart

weight increased from an average of 1.1 g on the day of injection to 1.55 g at 2 weeks.

The concentration of 8OHdG adducts to both nDNA and mtDNA of liver and heart following acute intoxication with doxorubicin is illustrated in Fig. 1. In all cases, the relative abundance (adducts per 10^5 dG nucleosides) was maximum at the earliest time examined (24 h). The concentration of adducts decreased exponentially thereafter, reaching control values by 1–2 weeks following the single injection of doxorubicin. The relative abundance of 8OHdG adducts to nDNA and mtDNA in control rats was between 1 and 4 per 10^5 dG for both liver and heart (data not shown). For both tissues at all time-points, the concentration of adducts to mtDNA was substantially higher than that for nDNA. For example, 24 h after an acute dose of doxorubicin, the ratio of mtDNA to nDNA adducts was 1.9 for liver and 2.2 for heart. Furthermore, the concentration of 8OHdG adducts was, without exception, higher in heart compared to liver. This was true for both nDNA and mtDNA adducts.

The time-dependent decline in the relative abundance of nDNA adducts was not due to dilution with native genomic DNA, as would occur with cell pro-

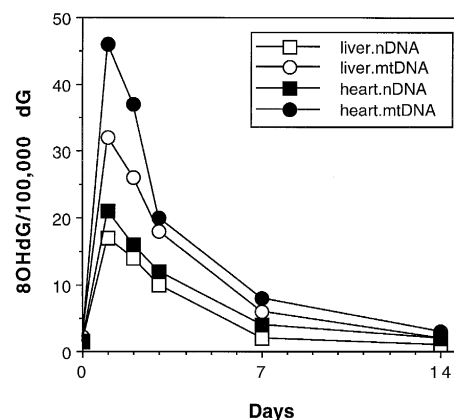


Fig. 1. Temporal changes in the relative abundance of 8OHdG adducts to both mtDNA and nDNA from heart and liver of doxorubicin intoxicated rats. Rats were injected i.p. with a single dose of 15 mg/kg doxorubicin. Mitochondria and nuclei were isolated by differential centrifugation and the DNA isolated and analyzed by LC/ESI/MS/MS for 8OHdG adducts. The concentration of the adducts is expressed in terms of unmodified dG nucleosides. Each point represents the mean of duplicate experimental samples. The standard deviation between samples was less than the diameter of the corresponding symbol.

liferation. The evidence is that the peak area response was very consistent among samples of nDNA (Fig. 2). Since a constant fraction of the total isolated DNA was analyzed each time, the peak area response provides a quantitative measure of total sample dG nucleosides. As illustrated in Fig. 2, peak area response for liver or heart nDNA from either control or treated animals did not change appreciably over time. In contrast, the peak area response of dG in both heart and liver mtDNA increased approximately 50% and 30%, respectively, within 3–7 days of the acute injection of doxorubicin (Fig. 2). This measure of total mtDNA remained elevated 2 weeks following doxorubicin intoxication.

The preferential accumulation of mtDNA adducts

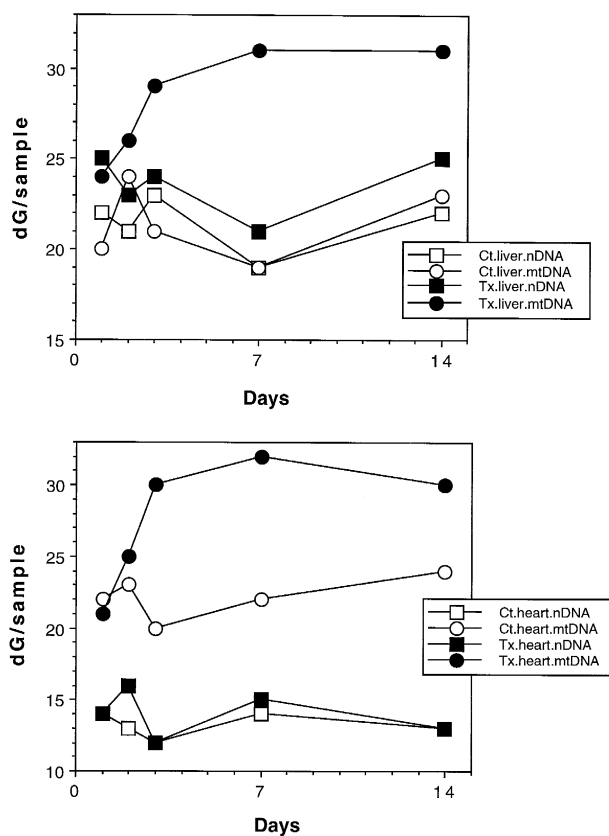


Fig. 2. Concentration of total unmodified deoxyguanosine (dG) bases in both liver (upper panel) and heart (lower panel) from control (Ct) and doxorubicin intoxicated (Tx) rats. dG concentration is expressed as the MS response factor on the basis of sample volume injected, which was always 4% of the total sample recovered from 1.5 g of tissue. Each point represents the mean of duplicate experimental samples. The standard deviation was less than the diameter of the respective symbols.

in vivo has been demonstrated following acute intoxication of rats or hamsters with various genotoxic agents [10,15–18,31]. There are a number of factors that may account for this. It may be that the hydrophobic xenobiotics preferentially partition to the lipid enriched mitochondrial membranes [12,14,15]. The close physical association of mtDNA to the inner mitochondrial membrane, which harbors the metabolic enzymes required for drug activation, favors the formation of mtDNA adducts. The absence from mitochondria of histone and non-histone proteins, which sheath the nDNA by providing a diffusional barrier to reactive genotoxic intermediates, also favors the formation of mtDNA adducts [32–34]. Finally, mitochondria lack the proficient excision- and recombination-DNA repair mechanisms characteristic of the nuclear genome [8–11]. Therefore, once formed there is a higher probability for the retention of unrepaired DNA adducts in mitochondria.

It is interesting to note the greater accumulation of adducts in cardiac tissue compared to liver, despite the fact that the rate of metabolic bioactivation of doxorubicin by cardiac microsomes is only 10–15% that of hepatic microsomes [35]. We are left to conclude either that the rate of metabolic activation of doxorubicin by microsomal enzyme systems is not a limiting factor or that comparisons of rates of drug metabolism measured in vitro do not accurately reflect that which occur in vivo. Alternatively, it is well established that doxorubicin undergoes rapid univalent reduction by NADH:ubiquinone oxidoreductase of the mitochondrial electron transport chain to initiate a futile redox cycle, liberating oxygen free radicals in the immediate vicinity of the mitochondrial genome [23,24]. Since mitochondria constitute up to 50% of the cell volume in myocytes, it could be that the principal site of metabolic activation and free radical generation in heart is the mitochondria, not the endoplasmic reticulum.

Regardless, the preferential accumulation of mtDNA adducts, especially in heart tissue, following acute intoxication with doxorubicin may account for many of the bioenergetic deficits associated with the cardiotoxicity observed in vivo. Such effects include the depletion of ATP, inhibition of both NADH- and succinate-cytochrome *c* reductase activities of the electron transport chain, and inhibition of respiratory

control by ADP [36–38]. These bioenergetic deficits are observed in mitochondria isolated from heart, but not liver, of intoxicated rats [39]. Since the mitochondrial genome provides exclusive encoding for 13 of the more than 80 proteins of the respiratory chain, mutations to the mtDNA, if unrepaired or not replenished, would result in an irreversible bioenergetic deficit. This is consistent with the cumulative and irreversible decline in cardiac performance observed clinically with doxorubicin.

The temporal dilution of 8OHdG adducts within both the mitochondrial and nuclear DNA of heart and liver suggests either rapid repair or amplification of the respective genomes. The time-dependent decline in relative abundance of nDNA adducts was expected because of the well characterized and efficient genomic repair mechanisms localized within the nucleoplasm. However, because of the virtual absence of mitochondrial DNA repair mechanisms [8–11], we did not expect to observe the resolution of the mtDNA adducts. One could suggest that cell hyperplasia might explain the dilution of mtDNA adducts in liver, but not heart. However, the fact that we did not observe an increase in total nuclear dG nucleotides (i.e., DNA content) indicates that the degree of toxic tissue injury, and thus the dose, was insufficient to elicit a mitogenic response. What was observed, though, was amplification of both the cardiac and hepatic mitochondrial genomes following acute intoxication with doxorubicin. Whether this reflects an increase in mitochondrial number or an increase in gene copy per individual mitochondrion remains unknown. The fact that we did not observe a treatment-related increase in either cytochrome oxidase or citrate synthase activities over this 2-week period (data not shown) favors an increase in mtDNA copy number as opposed to a stimulation of mitochondrial biogenesis. Although the time-course for genomic amplification correlates with the dilution of mtDNA adducts, it does not quantitatively account for the 5–6-fold dilution of 8OHdG adducts observed over the first week post-treatment.

Amplification of the mitochondrial genome occurs in response to a number of metabolically stressful situations such as exercise, hypoxia or hypertension and is essential to accommodating the exaggerated metabolic demands. Despite the importance of this phenomenon, very little is known regarding the pre-

cise signal that regulates mitochondrial biogenesis. Whatever the signaling pathway is, it apparently is responsive to the metabolic challenge associated with acute doxorubicin intoxication.

In conclusion, we suggest that the tissue and mitochondrial specific accumulation of 8OHdG adducts may be important in the phenotypic manifestation of bioenergetic deficits accompanying the cardiomyopathy associated with doxorubicin administration *in vivo*. Prevention of this adduct formation, potentiation of further genomic amplification, or development of therapies for circumventing the mitochondrial bioenergetic deficits may provide important opportunities for improving the clinical success of this important therapeutic regimen for treating a host of human cancers.

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