Induction by Carcinogens and Chemoprevention by N-Acetylcytstein of Adducts to Mitochondrial DNA in Rat Organs

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ABSTRACT

Damage to mitochondrial DNA (mtDNA) has been postulated to be associated with aging, cancer, and other chronic degenerative diseases which are the predominant causes of death in the population. We used molecular dosimetry techniques, i.e., $^{32}$P postlabeling and synchronous fluorescence spectrophotometry, in order to evaluate the formation of adducts to both mtDNA and nuclear DNA (nDNA) in different organs of rats exposed to genotoxic carcinogens. Adducts to mtDNA were detected following administration of benz(a)pyrene i.p. or 2-acetylaminofluorene by gavage as well as following exposure of animals to cigarette smoke. mtDNA adduct levels were consistently higher than those to nDNA in the same cells, and qualitative differences were also pointed out in the case of the aromatic amine. The oral administration of the thiol N-acetylcysteine, one of the most promising cancer chemopreventive agents endowed with nucleophilic and antioxidant properties, produced a significant decrease of mtDNA adducts in the liver of 2-acetylaminofluorene-treated rats and, sharply, in the lung and liver of rats exposed to cigarette smoke.

INTRODUCTION

mtDNA is a compact (16,569 bp), double-stranded, closed circular molecule (1) that is inherited maternally (2). mtDNA is more vulnerable than nDNA for a number of reasons, including the lack of protective histones and non-histone proteins, its proximity to oxidants generated during phosphorylative oxidation, a lower efficiency in DNA repair mechanisms, and a high rate of mitochondrial replication, which presumably removes the damaged organelles but renders mtDNA more susceptible to genotoxic agents (3, 4).

The findings that mitochondria are altered in cancer cells and that genotoxic carcinogens effectively bind mtDNA led to the hypothesis that mutations of this nucleic acid may be involved in the carcinogenesis process (3, 5). Moreover, a myriad of mutations and rearrangements in mtDNA have been implicated as a cause of mitochondrial dysfunctions, mainly as a result of defects in phosphorylative oxidation. These have been associated with aging processes as well as with a variety of chronic degenerative diseases, including some forms of ischemic heart disease, cardiomyopathies, adult-onset diabetes, Parkinson’s disease, Alzheimer’s disease, Huntington’s disease, and several other neurological disorders (6–10).

Due to the crucial role of mtDNA alterations in the pathogenesis of those clinical problems that are the predominant causes of death in developed countries, it is important to explore the mechanisms leading to mtDNA damage and to develop suitable preventative measures. With this aim, we used modern molecular dosimetry techniques, i.e., $^{32}$P postlabeling and SFS, for comparatively evaluating the formation of adducts to both mtDNA and nDNA in different organs of laboratory animals exposed to genotoxic carcinogens. These included two of the most extensively studied carcinogens, i.e., BP and 2AAF, which are typical representatives of the chemical families of polycyclic aromatic hydrocarbons and aromatic amines, respectively. These compounds are also typical components of complex mixtures, including cigarette smoke. Moreover, for the first time, we investigated molecular alterations of mtDNA following exposure to cigarette smoke itself. A major goal of the present study was to assess the protective effects of a pharmacological agent on the in vivo formation of DNA adducts. We used NAC, an analogue and precursor of reduced glutathione, the clinical efficacy and safety of which have been established in more than 30 years of experience as a mucolytic drug, in the therapy of respiratory diseases and, more recently, as an antidote toward several acute intoxications (11). Based on the experimental data generated by preclinical studies carried out during the past 12 years, NAC is now considered to be one of the most promising cancer chemopreventive agents and is currently being tested in several cancer chemoprevention clinical trials (12–15). NAC works through a variety of coordinated mechanisms, the most important of which depend on its remarkable antioxidant and nucleophilic properties, which warrant possible applications in the prevention of cancer and other pathological conditions associated with damage by free radicals (14, 15).

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (Morini strain), 3–4 months of age and weighing 220–260 g, were acclimatized for 7–14 days, maintained on a standard rodent chow (Morini, San Polo d’Enza, Italy), and given drinking water ad libitum. The animals were housed in a climatized environment at a temperature of 22 ± 1 °C, relative humidity of 50 ± 5%, and ventilation accounting for 15 air renewal cycles/h, and a 12-h light/dark cycle. Animal care was in accordance with our institutional guidelines.

Treatments. Each experimental group was composed of five rats. BP (Sigma Chemical Co., St. Louis, MO) was dissolved in DMSO and administered in a single i.p. injection (100 mg/kg body weight). Sham-exposed rats received an i.p. injection of the solvent. After 48 h, the animals were anesthetized with ethyl ether and killed by decapitation. Livers, lungs, and kidneys were removed.

2AAF (Sigma) was dissolved in olive oil and administered daily by gavage (100 mg/kg body weight) for 8 consecutive days. Sham-exposed rats received olive oil by gavage. After 48 h since the last treatment, the animals were killed, and the livers were removed.

A whole-body exposure of rats to cigarette smoke was obtained, as described previously (16), using filter-tipped commercial cigarettes having a declared content of 14 mg tar and 0.9 mg nicotine. The animals were exposed daily for 100 consecutive days to the mainstream smoke generated by eight cigarettes, accounting for a cumulative exposure of the rats composing this group to the smoke of 800 cigarettes. Sham-exposed rats were placed for the same time periods in an identical chamber but in the absence of cigarette smoke. After 24 h since the last exposure, the animals were killed, and livers and lungs were removed.

Additional groups of rats treated with 2AAF or exposed to cigarette smoke were co-treated with NAC in the form of a water-soluble commercial preparation containing 200 mg of this thiol (Fluimucil; Zambon Group, Vicenza, Italy). The drug was dissolved daily in drinking water at the concentration of 11 mg/ml, which was calculated to yield an average dose of 1 g NAC/kg body weight.
Chemicals were purchased from Sigma and Boehringer Mannheim GmbH.

DNA samples were digests. Furthermore, CsCl-ethidium bromide gradients provided further evidence that mtDNA was not contaminated with nDNA. DNA samples of the mtDNA molecule, i.e., supercoiled, relaxed circular, and linear, were obtained by solvent extraction, using an automatic DNA extractor (Genepure 341; Applied Biosystems, Foster City, CA), according to standard procedures (17), with some modifications as described previously (18).

The mitochondria were pellets by supernatant centrifugation at 9500 × g for 15 min. The pellets were washed twice with 0.25 M sucrose solution supplemented with 10 mM EDTA (pH 7.2–7.4) and resuspended in 2 ml of a buffer composed of 50 mM glucose, 10 mM EDTA, and 25 mM Tris-HCl (pH 8.0). mtDNA was purified by using an alkali extraction procedure, as described previously (19). As assessed by densiometric analysis of ethidium bromide-stained electrophoregrams, the yield of mtDNA, expressed as µg/g wet organ, was 4.1 for the kidney, 2.7–4.4 for the liver, and 1.2–1.9 for the lung. According to the electrophoretic patterns on agarose gel, all three forms of mtDNA molecule, i.e., supercoiled, relaxed circular, and linear, were present in the isolates from liver, lung, and kidney. The 260:280 nm ratio of the specimens tested was consistently higher than 1.7. No contamination by nDNA was detected when mtDNA preparations were tested by agarose gel electrophoresis of EcoRI, BamHI, HindIII, MspI, and HpaII restriction endonuclease digests. Furthermore, CsCl-ethidium bromide gradients provided further evidence that mtDNA was not contaminated with nDNA. DNA samples were stored at —80°C until use.

Detection of DNA Adducts. All samples were assayed in triplicate by 32P postlabeling and additionally, in the case of the BP experiment, by SFS. CHEMOPREVENTION OF ADDUCTS TO MITOCHONDRIAL DNA

Liver

Table 1 Adducts to nDNA and mtDNA in organs of rats treated with three carcinogens and effect of co-treatment with NAC

<table>
<thead>
<tr>
<th>Administered agent</th>
<th>Co-treatment with NAC</th>
<th>Rat organ</th>
<th>Adduct levels* (nDNA)</th>
<th>Adduct levels* (mtDNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP</td>
<td>—</td>
<td>Liver</td>
<td>SFSb</td>
<td>47.1 ± 7.6</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>Lung</td>
<td>SFSb</td>
<td>31.7 ± 5.7</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>Kidney</td>
<td>SFSb</td>
<td>7.3 ± 6.2</td>
</tr>
<tr>
<td>2AAF</td>
<td>—</td>
<td>Liver</td>
<td>32P postlabeling⁴</td>
<td>404 ± 29</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>Liver</td>
<td>Adduct 1</td>
<td>288 ± 37</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>Liver</td>
<td>(dG-C8-AAF)</td>
<td>Not tested</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>Liver</td>
<td>Adduct 2</td>
<td>116 ± 28</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>Liver</td>
<td>(dG-N2-AAF)</td>
<td>Not tested</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>Liver</td>
<td>Adduct 3</td>
<td>127 ± 47</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>Liver</td>
<td>(unknown)</td>
<td>Not tested</td>
</tr>
<tr>
<td>Cigarette smoke</td>
<td>—</td>
<td>Liver</td>
<td>32P postlabeling⁴</td>
<td>0.1 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>Liver</td>
<td>(Total adducts)</td>
<td>1.5 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>Lung</td>
<td>Undetectable</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>32P postlabeling⁴</td>
<td>11.7 ± 3.3</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>(Total adducts)</td>
<td>2.2 ± 0.9</td>
</tr>
</tbody>
</table>

* The results are means ± SD of three separate analyses.

b Results expressed as fluorescence units. dG-C8-AAF, deoxyguanosine-C8-aminofluorene; dG-N2-AAF, deoxyguanosine-N2-acetylaminofluorene.

* P < 0.01, mtDNA versus nDNA.

* Results expressed as adducts/10⁶ nucleotides.

* P < 0.05, mtDNA versus nDNA.

* P < 0.05, NAC+ versus NAC— (Student’s t test).

Results

Exposure to BP. The i.p. administration of BP to rats resulted in the formation of adducts to both mtDNA and nDNA, which were quantify by SFS (Table 1). The rank of adduct levels was kidney > liver > lung, with a mtDNA:nDNA ratio in these three organs of 1.4, 1.7, and 1.9, respectively. No fluorescent signal was detected when analyzing mtDNA or nDNA purified from the liver, lung, or kidney of sham-exposed rats receiving an i.p. injection of solvent. Fig. 1 shows an example of the results obtained at SFS by testing the liver mtDNA from sham-exposed rats or liver nDNA and mtDNA from BP-treated rats, displaying peaks of fluorescence with a maximum excitation peak at 351 nm.

When liver mtDNA from BP-treated rats was assayed by 32P postlabeling, three distinct adducts were detected, the patterns of which were identical to those produced by the corresponding nDNA samples, although the latter yielded a weaker signal (Fig. 2). As suggested by its chromatographic mobility (21), the major adduct (Fig. 2, no. 2) is likely to correspond to N²-[10β-(7,8,9,10-tetrahydro-7,8,9,10-tetrahydrobenzo(a)pyrene)]deoxyguanosine, whereas adduct no. 1 is presumably due to adduction to DNA of 4,5-epoxides of monohydroxylated metabolites in position 9 or 12 of the BP molecule (21). An adduct having the same chromatographic mobility as adduct no. 3 has been reported to be formed only in nDNA after in vivo exposure to BP and to be enhanced by treatment with cytochrome P-450 inducers (22).

Exposure to 2AAF and Co-Treatment with N-Acetylcycteine. Administration of 2AAF by gavage resulted in the formation of DNA adducts in rat liver, which were not detectable in sham-exposed rats.
animals. As shown in the example reported in Fig. 3, in addition to two common adducts, 32P postlabeling analyses consistently detected the presence of a third unidentified adduct to mtDNA that was absent in nDNA. According to their chromatographic mobility (23), adduct no. 1 is likely to correspond to deoxyguanosine-C8-AF, and adduct no. 2 to deoxyguanosine-\(N^2\)-AAF. In addition to this qualitative difference, the levels of adducts to mtDNA were higher than those to nDNA, i.e., 1.9-fold higher for adduct no. 1 and 1.4-fold for adduct no. 2. The total adduct levels were more than twice in mtDNA, compared to nDNA (Table 1).

The levels of DNA adducts were also measured in the liver mtDNA of 2AAF-treated rats receiving NAC with drinking water. All three adducts were decreased by administration of this thiol, the protective effect being statistically significant when comparing total adduct levels and adduct no. 2 (Table 1).

Exposure to Cigarette Smoke and Co-Treatment with NAC. The whole-body exposure of rats to mainstream cigarette smoke for 100 consecutive days resulted in the appearance of adducts to liver and lung (Table 1), which were detectable by 32P postlabeling in the form of typical diagonal radioactive zones (Fig. 4). The presence of adducts to liver nDNA was borderline to the sensitivity of the method. Adduct levels were considerably and significantly higher (\(P < 0.05\)) in the lung than in the liver for both nDNA (48-fold) and mtDNA (7.8-fold). Adducts to mtDNA were significantly higher (\(P < 0.05\)) than adducts to nDNA both in the liver (15-fold) and in the lung (2.4-fold).

Administration of NAC with drinking water attenuated the formation of smoke-related DNA adducts (Table 1; Fig. 4). In fact, adducts to nDNA were no longer detectable in the liver and were decreased in the lung, although such a difference did not attain the statistical significance threshold. Adducts to mtDNA were significantly lower (\(P < 0.05\)) in the liver (about 3-fold) and even lower in the lung (about 5-fold) of NAC-treated rats, as compared to the animals exposed to cigarette smoke without a chemopreventive agent.

DISCUSSION

The present study led to several new findings, which may bear relevance to the pathogenesis of cancer and other chronic degenerative diseases as well as to their prevention. As assessed by molecular dosimetry techniques, which can be also applied to human biomonitoring, adducts to mtDNA were found to be formed in different organs of laboratory animals exposed to individual DNA-binding carcinogens of different chemical classes. Moreover, adducts to mtDNA were detected in organs of rats exposed to cigarette smoke, a complex mixture associated with a variety of important degenerative diseases, which is the leading cause of death in developed countries. In all monitored organs, adduct levels to mtDNA were consistently higher than those to nDNA of the same cells. Note that adduction to liver mtDNA was not accompanied by changes in cytosine methylation patterns, as shown by previously reported restriction analyses of the same specimens (19). Another novel finding of the present study was that the oral administration of a chemopreventive agent was successful in attenuating these molecular biomarkers of biologically effective dose.

In particular, adducts to both mtDNA and nDNA were higher in the lung than in the liver of rats exposed to cigarette smoke, as docu-
CHEMOPREVENTION OF ADDUCTS TO MITOCHONDRIAL DNA

Fig. 3. Examples of 32P postlabeling of adducts to either nDNA or mtDNA enriched by butanol extraction in the liver of rats receiving 2AAF by gavage for 8 consecutive days. The panels show tridimensional image analyses of autoradiographs exposed for 6 h at –80°C. The possible nature of adducts 1-3 is specified in the text.

mented by the formation of diagonal radioactive zones of different intensity. These adduct patterns are typically detected by 32P postlabeling in the nDNA of organs from humans and laboratory animals exposed to complex mixtures, including cigarette smoke (24, 25). In rats receiving an i.p. injection of BP, the rank of adduct levels was kidney > liver > lung, as assessed by SFS, a technique that specifically evaluates the addition of benzo(a)pyrene diol-epoxide to DNA (18, 26, 27). The selective localization of DNA adducts in different organs is the result of several factors including: (a) pharmacokinetics and, in particular, “first-pass” effects of xenobiotics or their metabolites; (b) local metabolism; (c) efficiency and fidelity of DNA repair; and (d) cell proliferation rate (14, 26). Thus, first-pass effects are likely to account for the high levels of DNA adducts in the lung of rats exposed whole-body to cigarette smoke, which mainly involves inhalation of this complex mixture (27). It should be additionally noted that, compared to the lung, the liver has an outstanding metabolic capacity but, at the same time, is quite efficient at DNA excision repair, which removes DNA adducts (28).

32P postlabeling autoradiographs revealed that formation of adducts to mtDNA and nDNA can differ not only quantitatively but also qualitatively. In fact, in addition to deoxyguanosine-8-aminofluorene and deoxyguanosine-N2-acetylaminofluorene, an unidentified adduct very close to the latter one was detected in the liver mtDNA but not in nDNA of 2AAF-treated rats. This mtDNA-specific adduct may be tentatively ascribed to some short-lived 2AAF metabolite formed in proximity of mitochondria or even within these organelles.

The adduct levels were consistently higher to mtDNA than to nDNA, in most cases, to a statistically significant extent, but they were never dramatic, varying between a minimum of 1.4-fold (kidney of BP-treated rats) and a maximum of 15-fold (liver of rats exposed to cigarette smoke). To this respect, the data available in the literature are rather controversial, depending on the experimental conditions and on the method used for the assessment of DNA binding, although they generally point to a greater capacity of genotoxic agents to bind mtDNA rather than nDNA. In fact, the earliest studies testing tritium-labeled BP and other polycyclic aromatic hydrocarbons in cultured mammalian cells showed that covalent binding was greater to mtDNA than to nDNA by a factor ranging from 4 to over 500 (29–31). In rats exposed to tritiated aflatoxin B, adduction to liver mtDNA was 3–4 times higher than adduction to liver nDNA (32), and the localization of the aflatoxin B, guanosine adduct was also found to be severalfold greater in liver mitochondria than in nuclei, as evaluated by morphometric analyses (33). Binding of radioactive N-methyl-N-nitrosourea was, on the average, 5-fold higher than binding to nDNA, both in vitro and in vivo (34). Moreover, liver mtDNA was alkylated more extensively than nDNA following administration of dimethylnitrosamine (34, 35), whereas alkylation of mtDNA and nDNA was similar following administration of methyl methanesulphonate (36). By using 32P postlabeling, adds to mtDNA in the liver of rats receiving the food-borne heterocyclic amines IQ and PhIP by gavage were not higher than those to nDNA, excepting the group of animals receiving multiple doses of PhIP (37). In mice receiving i.p. injections of the heterocyclic aromatic carcinogen 7H-dibenzoc[a,g]carbazole or its metabolites, the mtDNA:nDNA binding index was lower than 1 (38). A preferential binding of cisplatin to the mtDNA of CHO cells, accounting for a 6-fold higher incorporation compared to nDNA, was assessed by using a fluoroimmunooassay procedure (39).

Under our test conditions, no mtDNA or nDNA adduct was detected in the organs of sham-exposed rats. Of particular interest is the possibility of revealing, by means of suitable modifications of 32P postlabeling, the so-called I-compounds (40), which are likely to be associated with DNA damage caused by reactive oxygen species (41, 42) and accumulate in an age-dependent manner in both nDNA and mtDNA (41). Most I-compounds, possibly originated from extramitochondrial sources, are common to mtDNA and nDNA, but a cluster of compounds, referred to as M-compounds, are mitochondria specific, presumably arising from DNA-reactive electron carriers of the mitochondrial electron-transport chain (41).

In spite of its small size, mtDNA is a highly critical target because of the extraordinary importance of the biochemical processes accomplished in these organelles. Therefore, covalent modifications of mtDNA are likely to be an important step as a mechanism involved in aging.
cancer, and a variety of other chronic degenerative diseases (see "Introduction"). Primary prevention of these modifications can be pursued primarily by avoiding exposure to genotoxic agents as much as possible. Besides this prescriptive approach, growing attention is paid to the possibility of preventing chronic degenerative diseases via a prescriptive approach, i.e., by favoring the intake of protective factors and by fortifying the physiological defense mechanisms of the host organism (14).

Previous studies performed in our laboratory showed that the oral administration of NAC can significantly decrease the formation of adducts to nDNA and specifically: (a) in the liver of rats receiving 2AAF with the diet; (b) in the liver, lung, heart, and testis of rats receiving intratracheal instillations of BP; (c) in the liver, lung, tracheal epithelium, heart, aorta, testis, and kidney of rats exposed to cigarette smoke (14, 18, 26, 43); and (d) in the lung of rats receiving intratracheal instillations of air particulate extracts from a polluted environment (44).

Adducts to nDNA are usually associated with the earliest steps of the carcinogenesis process, but according to our working hypothesis they also may be associated with other chronic degenerative diseases, e.g., with cardiomyopathies when they are localized in the heart and with the atherogenic process when they are localized in the aorta, as inferred from studies not only in rodents (14, 18, 26) but also in humans (45). The herein-reported decrease by NAC of adducts to mtDNA in the liver of...
rats treated with 2AAF and the sharp decrease in the lung and liver of smoke-exposed rats confirm the broad-spectrum protective potentiality of this chemopreventive agent and provide evidence that it is feasible to attenuate chemically induced molecular alterations of mtDNA by means of a pharmacological agent.

REFERENCES


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