Carcinogenesis vol.21 no.5 pp.991–998, 2000

One-electron oxidation is not a major route of metabolic activation and DNA binding for the carcinogen 7H-dibenzo[c,g]carbazole in vitro and in mouse liver and lung

Heather V. Dowty, Weiling Xue, Kathy LaDow, Glenn Talaska and David Warshawsky1

Department of Environmental Health, University of Cincinnati, Cincinnati, OH 45267-0056, USA

1To whom correspondence should be addressed
Email: david.warshawsky@uc.edu

7H-Dibenzo[c,g]carbazole (DBC) is a potent multi-site, multi-species carcinogen present in a variety of complex mixtures derived from the incomplete combustion of organic matter. Like many carcinogens, DBC requires metabolic activation to an electrophilic species to exert its mutagenic and carcinogenic effects. One-electron oxidation, leading to the formation of radical cation intermediates, has been proposed as a mechanism of metabolic activation for DBC in vitro resulting in unstable DNA adducts. The purpose of this research was to determine whether one-electron oxidation is a mechanism of activation and DNA binding for DBC in vivo. Specific depurinating DBC–DNA adducts formed by one-electron oxidation were analyzed in mouse liver at 4 h following a single i.p. dose of 40 mg/kg of 11 Ci [14C]DBC. In addition to five previously published adduct standards, two newly identified adduct standards were characterized by mass spectrometry and NMR, namely DBC-6-N7-Ade and DBC-6-N1-Ade; however, neither was observed in mouse liver. Only the DBC-5-N7-Gua adduct was observed in mouse liver extracts at a level of 6.5 ± 1.8 adducts/10^6 nucleotides. In addition, the formation of AP sites and stable DBC–DNA adducts was analyzed in mouse liver and lung at 4, 12 and 24 h following a single i.p. dose of 0.4, 4 or 40 mg/kg DBC (n = 3/group). There was a distinct time– and dose–response of stable DBC–DNA adducts formed by one-electron oxidation or the resulting AP sites also occur through this route constitutes a minor percentage of the total adducts formed.

Introduction

One-electron oxidation has been proposed as a major mechanism of metabolic activation for a number of polycyclic aromatic hydrocarbons (PAHs) in initiating DNA damage, compared with other routes of activation, such as diol epoxide metabolites (1). This pathway results in the formation of radical cation intermediates which are electrophilic and bind to DNA (2). In addition, the DNA adducts formed by this route of activation are unstable leading to spontaneous depurination. While there is evidence to support a role for radical cations in vitro, relatively few studies have investigated the importance of one-electron oxidation leading to DNA damage in vivo (3,4).

7H-Dibenzo[c,g]carbazole (DBC) is a potent carcinogen that belongs to the class of N-heterocyclic polynuclear aromatic hydrocarbons formed during the incomplete combustion of organic matter. As such, it is a component of a variety of complex mixtures including tobacco smoke condensate, synthetic coal fuels, shale oil, polluted river sediments and coal tar (5). It is tumorigenic and carcinogenic in a number of different species including mouse, rat, hamster and dog (6). It is an extremely potent liver carcinogen producing mainly hepatocellular adenomas and carcinomas (7). Like many carcinogens, DBC requires metabolic activation to exert its mutagenic and carcinogenic effects (8). The metabolic profile of DBC is unique, resulting in the formation of primarily phenol intermediates (9). The 3-OH metabolite of DBC has been proposed as a proximate genotoxicant in the activation of DBC to bind to DNA (10). However, the low ionization potential, 7.3 eV (11) of DBC indicates that one-electron oxidation and the formation of radical cation intermediates is another possible mechanism of activation.

Chen et al. (12) demonstrated that DBC is activated by one-electron oxidation in vitro following microsomal or horseradish peroxidase (HRP) metabolism in the presence of DNA. In this system, DBC forms four DNA adducts which spontaneously depurinate. The adducts observed presumably arise from a radical cation with charge localization primarily at the 5-position of DBC, but also the 6-position, including DBC-5-N7-Gua, DBC-5-N7Ade, DBC-5-N3Ade and DBC-6-N7-Gua. Spontaneous depurination of these adducts would result in the formation of apurinic (AP) sites as the major type of DNA damage. While AP sites are the most frequent spontaneous lesion in DNA, they are also mutagenic in both prokaryotic and eukaryotic cells (13,14).

A number of stable DBC–DNA adducts are detected in the target tissues liver and lung by 32P-post-labeling (15). However, it is not known whether unstable DBC–DNA adducts formed by one-electron oxidation or the resulting AP sites also occur in these target tissues. In this study, the formation of specific depurinating DBC–DNA adducts formed by one-electron oxidation were analyzed in the target tissue liver. In addition, the resulting AP sites were compared with the level of stable DBC–DNA adducts detected in the target tissues liver and lung.

Materials and methods

Chemicals

7H-Dibenzo[c,g]carbazole was newly synthesized to >99% purity following the method of Katritzky and Wang (7). Aldehyde reacting probe (ARP)

Abbreviations: AP sites, apurinic sites; ARP, aldehyde reacting probe; DBC, 7H-dibenzo[c,g]carbazole; HRP, horseradish peroxidase; PAHs, polycyclic aromatic hydrocarbons.

© Oxford University Press
was purchased from Dojindo Inc. (Kumamoto, Japan). Deuterated-DMSO containing 0.1% tetramethylsilane (TMS) was purchased from Aldrich (Milwaukee, WI). All other reagents were purchased from either Fisher Scientific (Pittsburgh, PA) or Sigma Chemical Co. (St Louis, MO).

**HRP activation**

DBC was activated by HRP in the presence of calf thymus DNA. The reactions contained 20 mM Tris-HCl pH 7.4, 0.5 mg/ml calf thymus DNA, DBC (50 μM) or DMSO, 5.0 PUMl HRP and 0.5 mM H2O2. Reactions were initiated by the addition of H2O2 and incubated at 37°C for 1 h. The reactions were treated with 0.2 mg proteinase K for 1 h at 37°C. The DNA was then precipitated and resuspended in filtered dH2O. The DNA was then analyzed for AP sites and stable adducts by ARp slot blot assay and nuclease P1 enhancement (2P-post-labeling assay, respectively.

**AP site analysis**

The aldehyde reacting probe slot blot assay was utilized to detect AP sites by following the methodology described by Nakamura et al. (16). AP site standards were generously provided by Jun Nakamura (Chapel Hill, NC). Briefly, 15 μg DNA (sample or standard) were incubated in 1 mM ARP in PBS for 10 min. DNA was precipitated with cold ethanol and resuspended in Tris-EDTA buffer at 3 μg/100 μl. DNA (100 μl) was heat denatured at 100°C for 5 min, quickly chilled on ice and mixed 1:1 with 2 M ammonium acetate. The samples were immediately applied to a supported nitrocellulose membrane pressurized with 20 psi using a slot blot vacuum filter apparatus. The filter was rinsed in 5x SSC for 15 min at 37°C, dried and baked in a vacuum oven at 80°C for 1 h. The membrane was then blocked with 10 ml Tris-NC1 buffer containing BSA (20 mM Tris-HCl, 0.1 M NaCl, 1 mM EDTA, 0.5% casein, 0.25% BSA, 0.1% Tween-20, pH 7.5) at room temperature for 1 h. The membrane was then incubated in the same solution containing streptavidin-conjugated HRP at room temperature for 30 min. After rinsing the membrane with washing buffer (0.26 M NaCl, 1 mM EDTA, 20 mM Tris-HCl, 0.1% Tween-20, pH 7.5) for 15 min, enzymatic activity was measured using ECL detection reagents and visualized by incubating with film for 5–45 s. The resulting bands on film were quantitated by image analysis using a Chemi imager 4000 (Alpha Innotech) and Chemi imagen software.

**2P-post-labeling-nuclease P1**

Duplicate DNA samples were hydrolyzed to 3’-monophosphate nucleotides by incubating with 0.25 U micrococcal nuclease and 2.5 μg spleen phosphodiesterase at 37°C for 6 h. Each mixture was incubated with 10 μl nuclease P1 cocktail (10 μg/ml nuclease P1, 0.054 mM ZnCl2, 0.075 M NaAc) for 5 min. Each mixture was incubated with 5 μl post-labeling cocktail containing 1.5 μl PKN-buffer (200 mM Bicine, 100 mM DTT, 10 mM Spermidine, 100 μM MgCl2), 0.28 μl polymeruclidean kinase (10.7 U/ml) was heat denatured at 100°C for 1 h. The reactions were treated with 0.25 U micrococcal nuclease and 2.5 units of nuclease P1 for 1 h at 37°C. The DNA was then analyzed for AP sites and stable adducts by ARP slot blot assay and nuclease P1 enhancement (2P-post-labeling assay, respectively.

**Synthesis of adenine and guanine positional isomer adducts of DBC by iodine**

Fluorescence spectra

DBC–DNA adduct standard synthesis and identification

DBC–DNA adducts were synthesized by incubating with 0.25 U micrococcal nuclease and 2.5 units of nuclease P1 for 1 h at 37°C. The DNA was then analyzed for AP sites and stable adducts by ARP slot blot assay and nuclease P1 enhancement (2P-post-labeling assay, respectively.

**Fluorescence spectrometry**

Fluorescence spectra were analyzed using an Hitachi F-4500 fluorescence spectrophotometer. Fluorescence excitation spectra were recorded at an emission wavelength of 400 nm and fluorescence emission spectra were recorded at an excitation wavelength of 278 nm. Synchronous scans of both standard and unknown peaks were achieved using a delta wavelength (Δλ) corresponding to their individual Stokes shift.

**DNA isolation**

Liver or lung tissue was homogenized in 1% SDS–100 mM EDTA to which protease K (0.2 mg) was added and incubated for 5 h at 55°C (post-labeling) or 12 h at 4°C (ARP assay). RNAs A (0.24 mg) and RNase T1 (40 μg) were then added and the mixture incubated for 30 min at 37°C. The homogenate was cooled on ice followed by protein precipitation with 5 M ammonium acetate. The supernatant was transferred to a new tube and DNA precipitated.
with 1 vol 2-propanol. The DNA was then rinsed in 70% cold ethanol, dried and resuspended in ddH2O pH 7.0. The DNA concentration was adjusted to 0.5 µg/µl.

**DNA hydrolysis**

DNA (2 µg) was hydrolyzed to 3’-monophosphates using 2.5 µg calf spleen phosphodiesterase and 0.25 U micrococcal endonuclease. The hydrolysis mixture was incubated for 6 h at 37°C.

**32P-post-labeling-carrier-free**

The hydrolyzed DNA was labeled at the 5’ position using 200 µCi [γ-32P]ATP and 1.7 U polynucleotide kinase at pH 9.0 and incubated for 40 min at 37°C. Samples were applied to the origins of poly(ethyleneimine)-cellulose (PEI) plates. Chromatography was carried out as outlined above.

**Results**

**Comparison of stable adducts versus AP sites following one-electron oxidation activation of DBC in vitro**

In this study, the formation of AP sites as measured by the ARP slot blot method was compared with the level of stable DNA adducts detected by 32P-post-labeling following activation of DBC by HRP in vitro. (Figure 1) There was a dose-dependent increase in both AP sites and stable DBC–DNA adducts, indicating that the response was a function of the amount of DBC binding to DNA. A marked difference was observed between the number of stable DNA adducts versus the number of AP sites in all treatment groups (Figure 1). A 6–18 fold excess of stable adducts compared with AP sites were noted between concentrations of 1–100 µM DBC. These results are clearly different than other reports which demonstrate that activation of DBC in vitro by HRP metabolism leads to a ratio of 2:1 depurinating adducts to stable adducts as measured by fluorescence line narrowing spectroscopy and 32P-post-labeling, respectively (12).

**DNA adduct standards**

The synthesis of monosubstituted positional isomers of DBC and adenine or guanine was carried out by iodine oxidation. In addition to the four expected adenine adducts, two additional adducts were observed, namely DBC-6-N7-Ade and DBC-6-N1-Ade. In all, six adenine adducts were observed by HPLC separation and confirmed by retention times (Table I), MALDI-TOF mass spectrometry and 1H-NMR according to previously published data (12), including DBC-5-N7-Ade, DBC-5-N3-Ade, DBC-5-N1-Ade, DBC-6-N3-Ade. Several guanine peaks were observed by HPLC; however, only the DBC-5-N7-Gua adduct was confirmed by mass spectrometry and 1H-NMR.

**DBC-6-N7-Ade**

The spectra of this adduct suggest substitution between the 6 position of DBC and the N7 position of adenine (Figure 2B). The shielding effect of the NH2(Ade) resulting in a broad singlet at δ5.91 is indicative of substitution at the N7 position of adenine. The upfield shift of the 8H doublet coupled with an absence of shielding effect on the 4H doublet implies substitution at the 6 position.

**DBC-6-N1-Ade**

The characteristics of this adduct suggest substitution at the 6 position of DBC and the N1 position of adenine (Figure 2C).
Identification of DBC–DNA adducts in mouse liver

Liver extracts from mice (n = 3) treated with [14C]DBC (40 mg/kg; 11 μCi) were separated by analytical HPLC using an acetonitrile:water gradient. A minor peak was observed at 24.6 min that corresponds to the retention time (24.6 min) of the DBC-5-N7-Gua standard (Table I). This peak was observed in each of the treated animals (Figure 3A). When HPLC fractions of each liver extract were collected, radioactivity was associated with this peak (Figure 3B). The retention time of the unknown peak was the same as the standard, DBC-5-N7-Gua. Upon re-injection of the fraction and standard, separately, onto the acetonitrile:water gradient the same retention times were observed (Figure 4A). When this fraction was again injected on the methanol:water gradient, the retention time was identical to that of the standard, DBC-5-N7-Gua (Figure 4B). Comparison of fluorescence spectra of the DBC-5-N7-Gua standard and the unknown fraction gave identical results (Figure 5). Peaks corresponding to the other adduct standards were not observed in the liver extracts, but they may be below our limits of detection.

Dose/time–response of stable DBC–DNA adducts in mouse liver and lung

Two-dimensional TLC autoradiograms (Figure 6) depict liver and lung DBC–DNA adducts following a single i.p. administration of 0 or 40 mg/kg. As expected, seven stable adducts were detected in mouse liver whereas three were observed in lung following i.p. administration. Adducts were numbered to correspond to previously published reports (15); however, an additional adduct positioned adjacent to adduct 1 was observed (40 mg/kg; 11 μCi) for 48 h. Extracts were separated by HPLC using an acetonitrile:water gradient and monitored for (A) fluorescence intensity or (B) radioactivity. The arrow refers to the peak corresponding to the DBC-5-N7-Gua adduct.

A downfield shift of the 5H proton, upfield shift of the 8H proton in conjunction with an absence of shielding effect on the 4H doublet, indicate substitution at the 6 position of DBC. A deshielding effect on the 2H(Ade) proton resulting in a downfield shift and a shielding effect on the 6-NH2(Ade) resulting in resonance to a downfield position is indicative of substitution at the N1 position of adenine.

A downfield shift of the 5H proton, upfield shift of the 8H proton in conjunction with an absence of shielding effect on the 4H doublet, indicate substitution at the 6 position of DBC. A deshielding effect on the 2H(Ade) proton resulting in a downfield shift and a shielding effect on the 6-NH2(Ade) resulting in resonance to a downfield position is indicative of substitution at the N1 position of adenine.
One-electron oxidation

Dose/time–response of AP sites in mouse liver and lung following DBC administration in vivo

AP sites were observed in both liver and lung DNA (Figures 9 and 10). While there was not a clear dose–response in all treatment groups, the number of AP sites in liver DNA were elevated at both 4 and 12 h post dosing at the mid and high dose groups. A significant increase of AP sites in liver DNA was observed \((P \leq 0.05)\) following the 4.0 and 40.0 mg/kg DBC treatment groups at 4 and 12 h, respectively, post-dosing. A statistically significant increase in AP sites was not observed in the lung DNA of treated animals.

Discussion

The interaction between metabolic activation and DNA damage is important in understanding basic mechanisms leading to mutagenesis and carcinogenesis by genotoxic compounds. This study has investigated whether one-electron oxidation is a mechanism of metabolism for the carcinogen DBC, resulting in the formation of unstable DNA adducts and AP sites in vitro and in vivo. This is a novel pathway of metabolic activation that has been largely supported by Cavalieri et al. (2,12). The significance of this pathway is presumed to be related to the levels of unstable DNA adducts produced in target tissues such as mouse skin (3,4). The resulting DNA damage from an unstable DNA adduct is an AP site. While AP sites are the most frequent spontaneous lesion in DNA, they are also mutagenic (13,14). The results of this work indicate that one-electron oxidation of DBC leading to the formation of unstable DBC–DNA adducts occurs in vitro and in mouse liver. This is quantitatively a minor (0.4%) pathway of activation and DNA binding.

In this study, a 6–18-fold excess of stable DNA adducts compared with AP sites were noted following activation of DBC with HRP in vitro. These results are in direct contrast with other reports that suggest a 2-fold excess of depurinating DBC–DNA adducts compared with stable DNA adducts in vitro (12). While the components and timing of the in vitro reactions were similar to other studies (12), it is not clear as to why such a quantitative discrepancy exists. Certainly different methodologies were used to quantify the resulting DNA damage, i.e. measuring AP sites directly versus indirectly by quantitating specific depurinating DNA adducts. In addition, interlaboratory variability in absolute adduct levels exists with the \(32\)P-post-labeling assay and may help to explain some of the quantitative differences observed between other studies (12) and the data presented here. Overall, the data presented here support the idea that unstable DNA adducts may be a minor mechanism of DNA damage by one-electron oxidation compared with stable DNA adducts in vitro.

Synthesis of DBC adenine adducts by iodine oxidation yielded the expected products namely, DBC-5-N7-Ade, DBC-5-N3-Ade, DBC-6-N3-Ade, DBC-5-N1-Ade and DBC-5-N7-Gua. Two additional adducts were also observed including DBC-6-N7-Ade and DBC-6-N1-Ade. Interestingly, the 5 or 6 positions of DBC were the only positions bound to adenine or guanine by iodine oxidation. This may indicate that charge density is localized at either of these positions.

Radical cation intermediates have been proposed as the activated metabolites formed by one-electron oxidation for a number of different PAHs, including DMBA, \(B[\alpha]\)P, \(DB[\alpha,\beta]\)P and DBC (4,3,12,17). Of these compounds only \(B[\alpha]\)P has been examined both in vitro and in mouse skin, the major

---

**Fig. 5.** Comparison of fluorescence spectra of liver extract fraction and DBC-5-N7-Gua adduct standard. (A) Fluorescence excitation spectrum monitored at \(\lambda_{Em} = 400\) nm; (B) fluorescence emission spectrum monitored at \(\lambda_{Ex} = 278\) nm; (C) synchronous fluorescence spectrum is monitored using a \(\Delta\lambda\) that equals the \(\lambda_{Em}\) of the peak with the shortest wavelength – \(\lambda_{Ex}\) of the peak with the longest wavelength.

**Fig. 6.** Two-dimensional TLC autoradiograms of DBC–DNA adducts from mouse liver and lung. Mice were treated i.p. with 40 mg/kg DBC (B and D) or solvent control (A and C) and killed at 4 h post-dosing. Liver (C and D) and lung (A and B) DNA were analyzed by \(32\)P-post-labeling. Chromatograms were exposed to film for 2–18 h.
target organ for B[a]P-induced skin tumors (4). The major adducts observed in skin were the depurinating B[a]P–DNA adducts formed by a one-electron oxidation mechanism and not the diol-epoxide pathway of activation. These observations were intriguing because they contradicted a long held belief that the diol-epoxide pathway is the only route of activation for B[a]P. In addition, the implications of this work suggested that a very different type of DNA damage, namely AP sites, may contribute to the mutagenic and carcinogenic potency of B[a]P in mouse skin. The finding that DBC was activated by one-electron oxidation, not only by HRP but also by microsomal enzymes, suggested that this mechanism of activation may be occurring in the target tissues relevant to DBC.

Only the DBC-5-N7-Gua adduct was observed in mouse liver extracts at a level of 6.5 ± 1.9 depurinating adducts/10^6 nucleotides. It has been proposed that for many PAHs, the major depurinating DNA adducts may reflect mutation specificity. For example, the major B[a]P–guanyl adduct is presumed to be a major intermediate in production of G→T mutations. However, the major base-substitution mutation observed following DBC administration in mouse lung is an A→T transversion. This type of mutation does not support a role for the involvement of the unstable DBC-5-N7-Gua adduct observed in mouse liver. This does not exclude the possibility that mutations may arise from the resultant AP sites. For example, a wide array of mutations induced by AP sites are observed in eukaryotic cells, including base substitution, frameshift and deletion mutations (14). It is not known, however, whether the amount of damage produced by this mechanism would result in an increased frequency of mutations.

If one-electron oxidation participates in the activation of DBC in its target tissues, specific depurinating adducts and AP sites would be the primary DNA damage produced through this mechanism. AP sites are not detected by the method of ^32P-post-labeling analysis. This suggests that while stable DBC–DNA adducts are detected in the target tissues liver, lung and skin, other types of DNA damage may be occurring that cannot be identified by ^32P-post-labeling analysis.

The number of AP sites detected in mouse liver DNA...
following DBC administration was elevated above control levels for most treatment groups. However, in mouse lung, there was no significant difference between control and treated groups. This is unlike observations in cells following administration of MMS where a distinct dose–response was noted (16). Detecting AP sites in vivo, however, may be challenging since the repair of AP sites is thought to be a rapid process. If AP sites are being formed and also repaired at a rapid rate, then damage by this mechanism may not be measurable in vivo. However, significant differences were observed in mouse liver, indicating that the formation of excess AP sites is a plausible mechanism of DNA damage by DBC in vivo.

While the one-electron oxidation mechanism of metabolism was explored in this study it was also necessary to put this into the context of stable DBC–DNA adduct formation in vivo. DBC is carcinogenic in mouse liver following dermal administration and carcinogenic in lung and tumors in liver following i.p. administration of 5–40 mg/kg. This carcinogenic potency is reflected in the levels of DNA adducts detected by 32P-post-labeling in these tissues. The structural characteristic of these adducts is still unknown; however, a 3-OH metabolite has been proposed as the proximate metabolite leading to stable DNA adducts (10).

DBC–DNA adducts were observed in liver and lung by 32P-post-labeling. As expected, adduct levels were more intense in the liver than in the lung. At the 4 h time-point and 40 mg/kg dose group, the relative adduct labeling index ×106 of stable adducts in liver equaled 1843 ± 195. This level far exceeds the level of AP sites or the depurinating DBC-5-N7-Gua adduct observed at the same dose group and time-point. At the 40 mg/kg dose level and 4 h time-point, stable adducts detected by 32P-post-labeling contribute to 99.6% of total adducts measured, whereas depurinating adducts contribute only 0.4% (Figure 11).

The time-course of adduct formation was much like that expected for a bolus dosing regimen. The increase in adduct formation is an integration of absorption, distribution and metabolic activation following i.p. administration. At the highest dose, the rate of change appears to steadily progress up to 24 h, whereas the low and mid doses appear to be reaching a maximum. Previous studies indicate that total DBC–DNA adducts peak around 24–72 h in liver following i.p. dosing, followed by a biphasic decline over a period of days persisting for up to several weeks (18).

The relative amounts of AP sites, DBC-5-N7-Gua adduct and stable DNA adducts detected by 32P-post-labeling were compared in mouse liver at the 40 mg/kg dose group and 4 h time point. Stable adducts outnumber AP sites or the DBC-5-N7-Gua adduct by roughly 300:1 (Figure 11). This observation clearly demonstrates that the stable DBC–DNA adducts detected by 32P-post-labeling are a major type of DNA damage. The unstable adduct was detected; however, it occurs at levels much lower than stable adducts. This indicates that one-electron oxidation leading to the formation of unstable DNA adducts and AP sites is not a major mechanism of DNA damage by DBC.

Acknowledgements

We would like to thank Jun Nakamura for providing AP site standards; Mark Rance and Vajira Nanayakara for generating the NMR and MS data; Marlene Jaeger and Howard Shertzer for helpful discussions. This work was supported by NIH grants 2R01-ES04203-11, 5T32-ES07250, IP30-ES06096-07 and P01 ES05652-08.

References


Received April 26, 1999; revised December 28, 1999; accepted December 30, 1999