Evidence for the Involvement of a Bis-Diol-Epoxide in the Metabolic Activation of Dibenz[a,h]anthracene to DNA-binding Species in Mouse Skin

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Abstract

Dibenz[a,h]anthracene (DB[a,h]A) and its microsomal metabolites, trans-3,4-dihydro-3,4-dihydroxydibenz[a,h]anthracene (DBA-3,4-diol), trans-3,4:8,9-tetrahydro-3,4:8,9-tetrahydroxydibenz[a,h]anthracene, trans-3,4:10,11-tetrahydro-3,4:10,11-tetrahydroxydibenz[a,h]anthracene (DBA-3,4,10,11-bis-diol) and trans-3,4:12,13-tetrahydro-3,4:12,13-tetrahydroxydibenz[a,h]anthracene were each applied topically to mouse skin and the epidermal DNA isolated 24 h later. 32P-postlabeling analysis of each of the DNA samples was performed. DNA from mice treated with DB[a,h]A produced an adduct map on TLC consisting of one major and three minor adduct spots. A similar pattern of spots was produced by DBA-3,4-diol. No detectable DNA adducts were produced by trans-3,4:12,13-tetrahydro-3,4:12,13-tetrahydroxydibenz[a,h]anthracene, although a single, minor adduct spot was produced by trans-3,4:8,9-tetrahydro-3,4:8,9-tetrahydroxydibenz[a,h]anthracene. However, DBA-3,4:10,11-bis-diol was found to produce a major single adduct that comigrated on thin layer chromatography with the major adduct produced by both DB[a,h]A and DBA-3,4-diol. In addition, this adduct was present at a level 10 times higher than the corresponding adduct produced by treatment with the parent hydrocarbon. Coelution of the major adducts formed from DB[a,h]A and DBA-3,4-diol with that formed from DBA-3,4,10,11-bis-diol was also demonstrated on reverse-phase high performance liquid chromatography. Thus, we propose that, in mouse skin, the major pathway of DB[a,h]A activation to DNA binding products is via a 3,4-diol to the 3,4,10,11-bis-diol and ultimately to a bis-diol-epoxide (potentially the 3,4,10,11-bis-dihydriodiol-1,2-oxide).

Introduction

DB[a,h]A is widespread in the environment as a result of the incomplete combustion of organic matter and was the first, pure polycyclic aromatic hydrocarbon reported to be carcinogenic in experimental animals (1). As with other chemical carcinogens of this class, DB[a,h]A exerts its effects in biological systems only after metabolism to reactive intermediates. The interaction between such species and the nucleophilic centers in cellular DNA can result in the formation of covalent DNA adducts, a process generally considered to lead to an initiating event in tumorigenesis. Analysis and characterization of such adducts can provide information as to the nature of the ultimate reactive metabolite formed from DBA-3,4-diol might be an epoxide formed through the further metabolism of a bis-diol which they and others (15) had detected as metabolites. In the present study we have used the 32P-postlabeling technique to investigate the DNA adduct-forming ability of these metabolites and report the high binding activity of one of them, DBA-3,4,10,11-bis-diol, which we believe may play a key role in the bioactivation of DB[a,h]A in mouse skin.

Materials and Methods

Chemicals. All chemicals and materials were from previously mentioned sources (16), except T4 polynucleotide kinase, which was obtained from Cambridge Bioscience, Cambridge, United Kingdom, and [γ-32P]ATP, which was obtained from ICN Biomedicals, Ltd., Buckinghamshire, United Kingdom. DBA-3,4-diol was supplied by Fluka (Neu-Ulm, Germany). DBA-3,4-diol was prepared as described previously (17). DBA-3,4,8,9-bis-diol (1′H-NMR (400 MHz; acetone-d6), 6.15 (dd, 1, H2, J1,2 = 10.08 Hz, J2,3 = 2.35 Hz), 7.19 (dd, 1, H1, J1,2 = 10.08 Hz, J2,3 = 2.35 Hz), 7.72 (d, 1, H6, J5,6 = 8.49 Hz), 7.84 (d, 1, H5, J5,6 = 8.49 Hz), 7.92-7.95 (m, 2, H9, H10), 8.07 (pseudo s, 2, H12, H13, H1, J1,2 = 9.2 Hz), 8.82 (s, 1, H3, J1,2 = 9.2 Hz), 8.91 (s, 1, H4a), and of DBA-3,4,12,13-bis-diol (1′H-NMR (400 MHz; acetone-d6), D2O 8 4.37 (dt, 1, H3, J1,2 = 11.40 Hz), 4.51-4.55 (m, 1, H1, J1,2 = 9.68 Hz), 4.61-4.65 (m, 1, H1, J1,2 = 9.68 Hz), 4.74 (d, 1, H1, J1,2 = 11.40 Hz), 6.15 (dd, 1, H5, J5,6 = 10.08 Hz, J6,7 = 2.35 Hz), 7.19 (dd, 1, H1, J1,2 = 10.08 Hz, J2,3 = 2.24 Hz), 7.70-7.78 (m, 2, H2, H3, H4, H5, J5,6 = 8.49 Hz, 8.72 (d, 1, H5, J5,6 = 8.49 Hz), 7.92-7.95 (m, 1, H1, 8.23 (d, 1, H4a), 8.34 (s, 1, H3, J1,2 = 9.2 Hz) will be published elsewhere.

Animals. Male Parkes mice, 4-6 weeks old, were obtained from the National Institute for Medical Research, Mill Hill, London. The backs of the animals were shaved and the mice were treated topically with two consecutive...
daily doses of 0.4 μm of a solution in tetrahydrofuran (50 μl) of either DB[a]A, DBA-3,4-diol, DBA-3,4,8,9-bis-diol, DBA-3,4,10,11-bis-diol, DBA-3,4,12,13-bis-diol, or tetrahydrofuran alone (as control). Groups of 2–4 mice were sacrificed 24 h after the final treatment and the dorsal skin was removed and frozen at −70°C.

**DNA Isolation.** The dermal surfaces of the frozen skin samples were scraped with a scalpel before fragmentation and powdering of the epidermal layers was performed in liquid nitrogen (19). Thawed and powdered skin samples were then homogenized in 10 mM EDTA and the DNA isolated as described previously (20).

**32P-Postlabeling Analysis.** DNA samples (4 μg) were digested with micrococcal nuclease (0.14 units) and spleen phosphodiesterase (1.2 μg) overnight, before further digestion for 1 h with nuclease P1 (0.15 units) as described previously (16, 21, 22). Samples were then 32P-labeled by incubation with carrier-free [γ-32P]ATP (50 μCi) and T4 polynucleotide kinase (6 units) at 37°C for 30 min; the reaction was terminated by the addition of apyrase (21).

Resolution of 32P-labeled adducts was performed on PEI-cellulose TLC sheets (20 × 20 cm) using the following solvent vectors: D1, 1.0 M sodium phosphate, pH 6.0; D2, 5.3 M lithium formate and 8.5 M urea, pH 3.5; D3, 1.2 M lithium chloride, 0.5 M Tris-HCl and 8.5 M urea, pH 8.0; D4, 1.7 M sodium phosphate, pH 6.0. The presence of radiolabeled adducts on the chromatograms was detected by autoradiography using an Autograph two-dimensional radioactivity detector system (Oxford Positron Systems, Ltd., Oxford, United Kingdom).

HPLC analysis of 32P-labeled adducts was carried out according to the method of Pfau and Phillips (23) with a Waters HPLC system consisting of two 501 HPLC pumps, a 712 WISP autosampler, and a Berthold LB 507A HPLC radioactivity monitor. Gradient control and data processing were achieved with a Waters Datatation with Baseline 810 software. Adduct spots, excised from PEI-cellulose TLC plates, were shaken overnight in 400 μl of 4 M pyridinium formate, pH 4.5, and the solution was filtered and evaporated under nitrogen and the residue was redissolved in water before injection onto a Zorbax phenyl-modified reverse-phase column (particle size, 5 μm; inside diameter, 250 × 4.6 mm) with elution using a solvent of 0.3 M phosphate buffer with a methanol gradient, as described previously for the separation of DB[a]A-DNA adducts (24).

**Results**

**32P-Postlabeling Analysis by TLC.** 32P-Postlabeling analysis of skin DNA from mice topically treated with DB[a]A revealed a number of minor adduct spots (numbered 1–3) plus one major spot (marked 5; 0.03 fmol adduct/μg DNA, equivalent to 66% of the total DNA binding) on PEI-cellulose TLC (Fig. 1B). This finding was consistent with previously published results (25) which provided evidence that adduct spot 2 was derived from the binding of the anti-3,4-diol-1,2-oxide. Postlabeling of epidermal DNA from mice treated with DBA-3,4-diol resulted in chromatograms that were similar to that obtained with DB[a]A (Fig. 1C); adducts were detected at positions 1–3 plus an additional adduct spot was detected at position 4. Again, the major adduct produced by treatment with DBA-3,4-diol was at position 5 (0.04 fmol adduct/μg DNA). Analysis of skin DNA from mice treated with the three bis-diol derivatives of DB[a]A was also performed; DBA-3,12,13-bis-diol gave an entirely blank adduct map (Fig. 1D), DBA-3,4,8,9-bis-diol produced a single weak adduct spot at a position distinct from the major adducts generated by treatment with either DB[a]A or DBA-3,4-diol (Fig. 1E), and two adduct spots were produced by DBA-3,4,10,11-bis-diol (Fig. 1F). One of these adducts was relatively minor and migrated at position 1; however, a much more highly labeled adduct spot (0.41 fmol adduct/μg DNA) was detected at position 5. This adduct appeared to exhibit similar chromatographic properties to the major adduct generated by
treatment of mouse skin with either DB[a,h]A or DBA-3,4-diol and was present at a level 10-fold higher than the adduct formed by treatment with the parent hydrocarbon. In order to confirm the matching chromatographic identity of this adduct, cochromatography experiments with mixtures of DNA from (a) DB[a,h]A-treated and DBA-3,4,10,11-bis-diol-treated mouse skin and from (b) DBA-3,4-diol-treated and DBA-3,4,10,11-bis-diol-treated mouse skin were performed. The results demonstrate the comigration of the major DBA-3,4,10,11-bis-diol adduct with the major adducts derived from both DB[a,h]A and from DBA-3,4-diol (Fig. 1, G and H, respectively).

**32P-Postlabeling Analysis by HPLC.** The comigration of the major DNA adducts of DB[a,h]A, DBA-3,4-diol, and DBA-3,4,10,11-bis-diol on TLC, with its relatively low resolving power, warranted confirmation using an alternative chromatographic system such as HPLC. Fig. 2A shows the HPLC elution profile obtained with 32P-postlabeled DNA from DB[a,h]A-treated mouse skin following elution from the origin of a TLC plate after development in one dimension with solvent vector D1. The analysis by HPLC of this total adduct mixture in DNA from DB[a,h]A-treated mouse skin produced numerous peaks including a broad major peak beginning at 20.3 min which possessed shoulders at 24.0- and 26.0-min retention times (Fig. 2A).

The major adduct spot 5 that had been eluted following the multidirectional TLC separation of DB[a,h]A adducts (Fig. 1B) was found to give a peak with a retention time of 22.2 min on reverse-phase HPLC (Fig. 2B), a finding consistent with that published previously (24). Chromatography of the major adduct formed from treatment of mouse skin with DBA-3,4-diol also gave a peak with a similar retention time (21.8 min; Fig. 2C). Although a slight difference in retention times was apparent between these DB[a,h]A- and DBA-3,4-diol-DNA adduct peaks, this appeared to be due to slight fluctuations of the solvent composition during gradient elution, since the coinjection of the eluted major adducts of both DB[a,h]A and the DBA-3,4-diol was found to produce only a single peak at 21.2 min (Fig. 2D). HPLC of the DBA-3,4,10,11-bis-diol major adduct was also found to produce a single peak at a retention time of 21.2 min (Fig. 2E). Again, this adduct coeluted with both the DB[a,h]A-derived adduct (Fig. 2F; retention time, 21.4 min) and the DBA-3,4-diol-derived adduct (Fig. 2G; retention time, 21.4 min) when samples were coinjected. HPLC elution of the minor adduct produced by the binding of the DBA-3,4,8,9-bis-diol gave two peaks with retention times of 23.9 and 26.3 min, respectively (Fig. 2H). Although these adduct peaks were distinct from the major adduct formed by the binding of DB[a,h]A, as described above, minor peaks at similar retention times of 24.0 and 26.0 min were present in labeled hydrolysates of DNA from DB[a,h]A-treated mouse skin (Fig. 2A).

**Discussion**

It is now more than 60 years since DB[a,h]A was first shown to be carcinogenic in experimental animals (1). However, despite extensive work on the metabolism of this carcinogen, the definitive route of activation to the major DNA-binding species has proved elusive. When DB[a,h]A metabolism was investigated in microsomal incubations, the three possible diols were detected with the major diol metabolite being the 3,4-diol, the precursor of the simple bay region diol-epoxides (7, 8, 26) (Fig. 3). This diol was subsequently found to be a potent mutagen in mouse liver (23) and to be more tumorigenic in mice than the related 1,2- and 5,6-diols (12). Investigations into the biotransformation of the 3,4-diol yielded conflicting results. While some workers (8) obtained evidence in rodent hepatocyte preparations for extensive formation of 3,4-diol-1,2-oxides, others (14) detected these bay region diol-epoxides and their hydrolysis products in only small amounts. Recently, however, studies have demonstrated that the parent hydrocarbon and the 3,4-diol can be converted to a range of polyhydroxylated products including bis-dihydrodiols. Both the 3,4,10,11- and the 3,4,12,13-bis-diols were detected as microsomal metabolites of DB[a,h]A (9, 15), together with the 1,2,3,4,8,9- and 1,2,3,4,10,11-hexols, which may have arisen from the hydration of related diol-epoxides. While DBA-3,4,10,11-bis-diol was activated by the postmitochondrial fraction of Aroclor 1254-pretreated rats to even more potent bacterial mutagens than was its metabolic precursor, DBA-3,4-diol (14), DBA-3,4,12,13-bis-diol is only very weakly mutagenic. These results have led to the proposal that bis-diol-epoxides might be the ultimate reactive metabolites formed from both DB[a,h]A and DBA-3,4-diol. Thus in this report we have investigated this hypothesis by using the 32P-postlabeling technique coupled with TLC and HPLC to examine the nature of the adducts formed from DB[a,h]A and its metabolites. The results presented here show that, with the three bis-diols investigated, no DNA adducts were detected with DNA from mice treated with DBA-3,4,12,13-bis-diol and only a minor adduct spot was seen with DBA-3,4,8,9-bis-diol. This minor adduct spot produced two peaks (at

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*K. Platt, unpublished results.*

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Fig. 2. Reverse-phase HPLC separations of eluted adduct spots from TLC of 32P-postlabeled mouse skin DNA samples. A. DB[a,h]A treatment. Total adducts eluted from TLC plates run in D1 only. B. Adduct spot 5 from DB[a,h]A treatment. C. Adduct spot 5 from DBA-3,4-diol treatment. D. Coinjection of adduct spot 5 derived from DB[a,h]A and from DBA-3,4-diol. E. Adduct spot 5 from DBA-3,4,10,11-bis-diol treatment. F. Coinjection of adduct spot 5 derived from DB[a,h]A and from DBA-3,4,10,11-bis-diol. G. Coinjection of adduct spot 5 derived from 3,4,8,9-bis-diol and from DBA-3,4,10,11-bis-diol. H. Minor adduct spot derived from 3,4,8,9-bis-diol.
BIS-DIOL EPOXIDES IN THE METABOLIC ACTIVATION OF DIBENZ[a,l]ANTHRACENE

Fig. 3. Proposed pathways of metabolism of DB[a,h]A in mouse skin and activation to a diol-epoxide and a bis-diol-epoxide. For simplicity, epoxide intermediates in the formation of DBA-3,4-diol from the hydrocarbon and of the bis-diols from DBA-3,4-diol have been omitted.

～24 and 26 min) when eluted on HPLC and these peaks possessed similar retention times to two minor adducts from labeled hydrolysate of DB[a,h]A-treated mouse skin DNA. Further studies will be required to establish if the two minor adduct peaks seen with DNA from DB[a,h]A-treated mouse skin are in fact derived from the asymmetrical DBA-3,4,8,9-bis-diol. Treatment of mouse skin with DBA-3,4,11-bis-diol, however, produced a major DNA adduct and it is of interest to note that Platt and Reischmann (9) proposed that this particular bis-diol could produce, in theory, a very reactive bis-diol-epoxide. The major adduct formed from DBA-3,4,11-bis-diol cochromatographed both on TLC and HPLC with the major adduct formed following treatment with DB[a,h]A or DBA-3,4-diol. Furthermore, adduct formation was 10-fold higher with this bis-diol than with the precursor diol or parent hydrocarbon. Hence we suggest that this particular symmetrical bis-diol may represent the penultimate metabolite in a pathway by which a bis-diol-epoxide of the bay region type (Fig. 3) is formed as the ultimate DNA binding species. This route is not, however, exclusive since we have previously provided evidence that the minor adduct spot 2 (formed by DB[a,h]A and DBA-3,4-diol) is derived from the binding of the DB[a,h]A anti-3,4-diol,1,2-oxide (25). It is not known, at present, why the 3,4-diol is not converted rapidly to simple diol-epoxides. It is conceivable that the monoxygenase finds that the unhydroxylated lipophilic benzene ring at the opposite end of the molecule is a better alternative to the vicinal olefinic double bond, thus giving rise to a bis-diol. Since DBA-3,4,10,11-bis-diol is symmetrical and since both diol moieties have isolated olefinic double bonds adjacent to a bay region, its conversion to a bis-diol-epoxide where the diol-epoxide grouping is of the bay region type can be envisaged. Definitive proof that the major ultimate DNA-binding metabolite derived from DB[a,h]A is a bis-diol-epoxide of this type must await synthesis and physico-chemical characterization of the bis-diol-epoxides and their resultant DNA adducts.

References

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