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The In Vivo Formation and Repair of DNA Adducts From 1′-Hydroxysafrole

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1′-Hydroxysafrole is a proximate carcinogenic metabolite of the naturally occurring hepatocarcinogen safrole. Comparison by high-performance liquid chromatography of the nucleoside adducts obtained from hepatic DNA of adult female mice treated with [2′,3′-^{3}H]1′-hydroxysafrole with those formed by reaction of deoxyribonucleosides with electrophilic derivatives of 1′-hydroxysafrole indicated that the four in vivo adducts studied were derived from an ester of 1′-hydroxysafrole. Three of the four adducts comigrated with products of the reaction of 1′-acetoxysafrole with deoxyguanosine, whereas the fourth adduct comigrated with the major reaction product of the ester with deoxyadenosine. Analysis of the three deoxyguanosine adducts indicated that all three involve substitution on the 2-amino group of guanine. A sample of the major adduct prepared from deoxyguanylic acid has been characterized from its NMR spectrum as N^2-(*trans*-isosafrol-3′-yl)-deoxyguanosine, and the deoxyadenosine adduct has been similarly characterized as N^6-(*trans*-isosafrol-3′-yl)-deoxyadenosine.

Repair replication was measured in cultured human T98G cells exposed to 1′-acetoxysafrole using the combined 5-bromodeoxyuridine density label and radioisotopic label method. At a concentration of 1 mM 1′-acetoxysafrole, the amount of repair synthesis approached maximum values only about 15% of those obtained after saturating doses of ultraviolet light. Repair patch size distribution was found to be similar in cells treated with ultraviolet light or 1′-acetoxysafrole as determined by the density of repair-labeled DNA relative to that of parental DNA.

Key words: safrole, 1′-hydroxysafrole, 1′-acetoxysafrole, carcinogen-DNA adducts, DNA repair, 5-bromodeoxyuridine density labelling, repair patch size

Safrole (1-allyl-3,4(methylenedioxy)benzene) belongs to a large class of ring-substituted allylbenzenes and propenylbenzenes, which occur in many higher plants that are used in the cosmetic and food flavoring industries [1]. When fed to adult rats or mice [2–4] or injected into neonatal mice [3–5], safrole displays weak hepatocarcinogenic activity.

Abbreviations: UV, ultraviolet light (254 nm); HPLC, high-performance liquid chromatography; dThd, thymidine; PBS, phosphate-buffered saline; DMSO, dimethylsulphoxide; BrdUrd, 5-bromodeoxyuridine; FdUrd, 5-fluorodeoxyuridine; dGuo, deoxyguanosine; dAdo, deoxyadenosine.

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A current concept in chemical carcinogenesis is that most carcinogens exert their biological effects via the covalent interaction of electrophilic species with cellular macromolecules [6]. In the case of safrole, the metabolic formation of several electrophilic species is indicated [4, 7–13]. 1′-Hydroxysafrole is a major metabolite of safrole in the rat and mouse, and is a stronger carcinogen than the parent compound in these species [3, 8], which suggests that it is an intermediate in the metabolic activiation of safrole. Rat and mouse liver preparations convert 1′-hydroxysafrole to 1′-sulphonoxysafrole and 1′-hydroxysafrole-2′,3′-oxide [11, 13] (Fig. 1), and both the latter metabolite and an analog of the former, 1′-acetoxysafrole, possess electrophilic, carcinogenic and mutagenic activity [3, 4, 8, 11, 12]. Another metabolite, 1′-oxosafrole [7] (Fig. 1), also exhibits electrophilic activity [11], but no demonstrable mutagenic or carcinogenic activities [4, 11].

1′-Hydroxyestragole, a major metabolite of the structurally related carcinogen estragole (1-allyl-4-methoxybenzene) [14], has been shown to be metabolically activated in mouse liver via a 1′-ester, which reacts covalently with the N^2-atom of guanine and the N^6-atom of adenine residues in DNA [15]. The present paper summarizes the evidence to date for the structures of the DNA adducts formed by metabolically activated 1′-hydroxysafrole in mouse liver in vivo, and reports on the response in cultured human cells to DNA damage by 1′-acetoxysafrole. In mouse liver, the DNA adducts formed are closely analogous to those reported for 1′-hydroxyestragole [15]. In cultured T98G cells, DNA damage due to 1′-acetoxysafrole is repaired with a patch size distribution similar to that found for UV-induced damage, as determined by the density of repair-labeled DNA relative to that of parental DNA [16].

MATERIALS AND METHODS

Chemical Syntheses

Safrole derivatives were synthesized by previously described methods [8, 11]. Marker nucleoside adducts were prepared by reacting the appropriate 1′-hydroxysafrole derivative with ^{14}C-labeled nucleosides as described earlier [15].

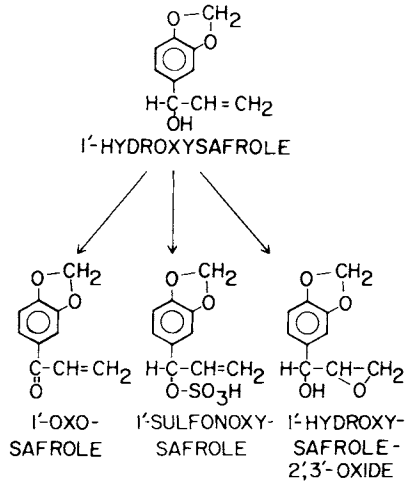

Fig. 1. Demonstrated pathways of metabolism of 1′-hydroxysafrole, a proximate carcinogenic metabolite of safrole, to electrophilic species.

Animal Experiments

Female CD-1 mice, 8–10 weeks old, mean weight 30 g, were injected IP with (2′,3′-^{3}H)1′-hydroxysafrole (404 mCi/mmol, 12 μmol/mouse in 0.1 ml trioctanoin). The animals were killed 23 hours after treatment, and their liver DNA was isolated and hydrolyzed enzymatically to nucleosides as described previously [15].

Chromatography

HPLC chromatography of DNA hydrolysates from mouse liver and of marker nucleoside adducts was performed on an ALC/GPC 204 liquid chromatograph (Waters Associates, Milford, Massachusetts) equipped with a model U6K injector, a model 660 solvent programmer, and a Spherisorb ODS 5 μ reverse-phase column (Altex Scientific, Berkeley, California). The solvent system was 0–5 minutes, 100% water; 5–40 minutes, 15–40% acetonitrile/water (linear gradient). The flow rate was 2 ml/min.

Cells

T98G cells, derived from a human glioblastoma tumour [17], were grown in minimal essential medium containing 10% fetal calf serum. Cells that had been prelabeled with (^{14}C)dThd were grown to confluence and treated with 1′-acetoxysafrole in 100:1 PBS:DMSO. Because of the short half-life (less than 2 minutes) of 1′-acetoxysafrole in aqueous solution, cells were layered with PBS, and the compound was dissolved in DMSO and administered directly to the culture dish with immediate stirring. During the 25-minute treatment period the PBS was removed and replenished twice, and fresh compound was administered each time. After treatment the cells were washed thoroughly with PBS.

DNA Repair Determinations

Repair replication was examined using the combined BrdUrd density label and radioisotopic label method [16, 18]. Cells were incubated posttreatment for 4 hours in medium containing 1 μM FdUrd, 10 μM BrdUrd, and 10 μCi/ml (^{3}H)dThd (79.4 Ci/mmol). By means of equilibrium sedimentation in CsCl gradients, the short stretches of DNA synthesis due to repair can be distinguished from extensive chain elongation by residual semiconservative replication. Thus repair replication is measured by the incorporation of (^{3}H)dThd into unreplicated parental density DNA. After the incubation with (^{3}H)dThd and BrdUrd, the cells were lysed and sedimented in neutral CsCl gradients, from which the fractions containing parental density DNA were pooled and rebanded in alkaline CsCl gradients [16]; fractions were collected and assayed for radioactivity.

Repair Patch Size Determination

If the molecular weight of the DNA is reduced to the extent that density-labeled repair patches have measurable effects on the density of the DNA fragments containing them, then the above method can be used to analyze the size distribution of the patches [16]. After treatment with UV or 1′-acetoxysafrole, cells were incubated in medium containing 10 μM FdUrd, 100 μM BrdUrd, and 10 μCi/ml (^{3}H)dThd, and their parental density DNA was purified on neutral CsCl gradients. The DNA was then sonicated before analysis in alkaline CsCl gradients [16]. The average size of the DNA fragments was determined separately by velocity sedimentation in alkaline sucrose gradients.

RESULTS

The HPLC elution profiles of DNA hydrolysates from the livers of mice injected with (2′,3′-^{3}H)1′-hydroxysafrole are shown in Figure 2. In the later regions of the chromatographs, four well-defined peaks eluted; these are designated I–IV in order of elution. Significant amounts of radioactivity also eluted in the early and intermediate regions, but, apart from the region where unmodified nucleosides elute (9–12 minutes), no well-defined peaks were discernible. When hepatic DNA hydrolysates were coinjected on HPLC with an aliquot of the reaction mixture of 1′-acetoxysafrole with (^{14}C)dGuo, ^{14}C-labeled products eluted with the ^{3}H-labeled in vivo adducts I, II, and III (Fig. 2A). Adduct IV comigrated with a ^{14}C-labeled product from the reaction of 1′-acetoxysafrole with (^{14}C)dAdo (Fig. 2B). As described elsewhere [19], these comigrations of in vivo and in vitro adducts were also observed with other solvent systems, whereas the products of reactions of 1′-hydroxysafrole-2′,3′-oxide and 1′-oxosafrole with dGuo and dAdo did not comigrate with any of the adducts I–IV. Some of them did, however, elute in regions of the chromatograph containing low levels of ^{3}H (15–22 minutes). Thus, the adducts studied arise in mouse liver DNA in vivo from 1′-hydroxysafrole via an electrophilic ester (or a metabolite with equivalent reactivity), but the minor involvement of other pathways of activation cannot be excluded.

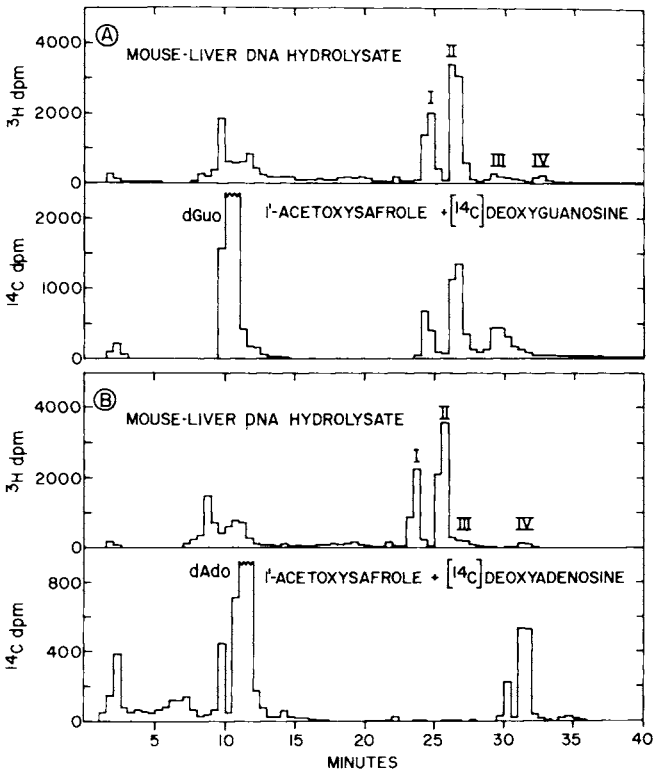

Fig. 2. Reverse-phase HPLC profiles of DNA hydrolysates and marker nuclosides. DNA hydrolysates from the livers of mice injected with (^{3}H)1′-hydroxysafrole were cochromatographed with aliquots of the reactions mixtures of 1′-acetoxysafrole reacted with: A, (^{14}C)dGuo; B, (^{14}C)dAdo.

Analysis of the dGuo adducts, described in detail elsewhere [19], by determination of their pH-partition coefficient patterns and the retention of ^{3}H by the adducts formed from the reaction of 1′-acetoxysafrole with (8-^{3}H,8-^{14}C)dGuo indicated that all three adducts contain guanine residues substituted on the exocyclic 2-amino group. Synthetic samples of adduct II, the major dGuo adduct, and adduct IV, the dAdo adduct, were obtained by large scale reaction of 1′-acetoxysafrole with the appropriate deoxyribonucleotide followed by enzymatic dephosphorylation, and characterized from their NMR spectra as N^2-(*trans*-isosafrol-3′-yl)-deoxyguanosine and N^6-(*trans*-isosafrol-3′-yl)-deoxyadenosine, respectively [19]. The structures of these adducts are shown in Figure 3. Adducts I and III have not yet been further characterized.

DNA repair synthesis in human T98G cells after treatment with different doses of 1′-acetoxysafrole is shown in Figure 4. Incorporation of (^{3}H)dThd into parental density DNA in the 4 hours following treatment approached maximum values with a concentration of 1 mM 1′-acetoxysafrole. At higher concentrations (2–3 mM 1′-acetoxysafrole), there was slightly less DNA repair in 4 hours than at the 1 mM level, indicating that DNA repair may be inhibited at these high concentrations of the compound. At the 1-mM level, the amount of repair synthesis in 1′-acetoxysafrole-treated cells was approximately 15% of that occurring in cells that had been treated with a saturating dose (with respect to repair synthesis) of 50 J/m^2 UV.

The repair patch size distribution in UV- and 1′-acetoxysafrole-treated cells was compared as described in Materials and Methods. The size of the DNA isolated after purification and sonication was determined by sedimentation velocity on alkaline sucrose gradients to have a number average of about 200 nucleotides. The density shift of the repair-labeled DNA from the prelabeled parental density DNA in alkaline CsCl gradients is shown in Figure 5. For both damaging agents, the profiles are indicative of "short-patch repair," with an estimated patch size of 30 nucleotides for UV and 24 nucleotides for 1′-acetoxysafrole.

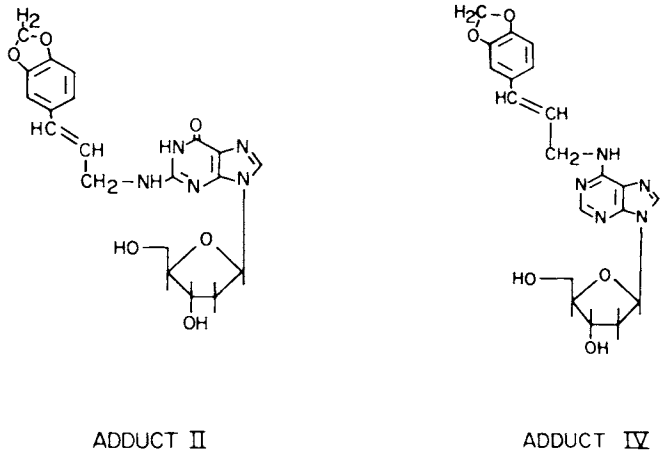

Fig. 3. Structures of the major dGuo and dAdo derivatives formed from reaction of 1′-acetoxysafrole with the mononucleotides. These adducts comigrated on HPLC with adducts isolated from the DNA of mice administered 1′-hydroxysafrole.

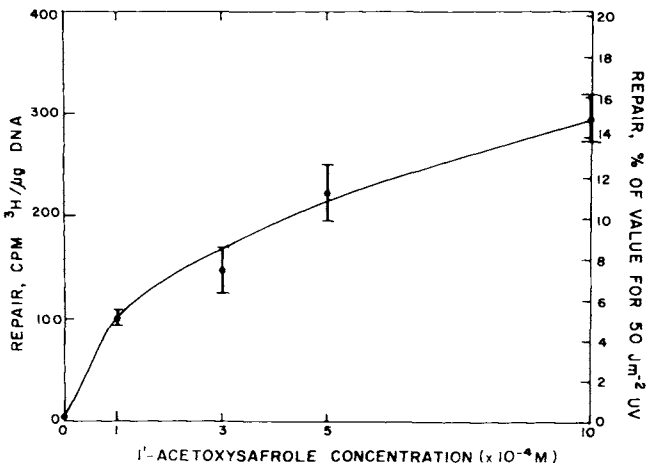

Fig. 4. DNA repair in human T98G cells in 4 hours following treatment with 1′-acetoxysafrole. Repair replication (labeled with (^{3}H)dThd) was distinguished from semiconservative normal replication by BrdUrd density labeling and isopycnic sedimentation in CsCl density gradients. Results of duplicate experiments are shown.

DISCUSSION

The principal DNA adducts formed in mouse liver after administration of 1′-hydroxysafrole have been characterized as containing N^2-substituted guanine and N^6-substituted adenine residues. Both the major dGuo adduct (II) and the dAdo adduct (IV) are *trans*-isosafrole derivatives substituted at the 3′ position. While adducts I and III have not been characterized beyond determining the site of substitution on the guanine residue, the close similarity of the HPLC profiles between the 1′-hydroxysafrole adducts (Fig. 2) and those formed from 1′-hydroxyestragole [15] suggests that adducts I and III are analogous to the 1′-hydroxyestragole adducts I and III. Evidence has been presented that the latter two adducts are N^2-(estragol-1′-yl)-deoxyguanosine and N^2-(*cis*-isoestragol-3′-yl)-deoxyguanosine, respectively. The formation of adducts with these different sites of substitution on the estragole and safrole moieties can be accounted for by the reaction of an electrophile, presumably a 1′-ester, with purine bases in DNA by either S_N1 or S_N2 and S_N2' mechanisms [15, 19].

The similarity between the adducts formed by 1′-acetoxysafrole reacted with deoxyribonucleosides and by 1′-hydroxysafrole in mouse liver DNA in vivo suggests that 1′-acetoxysafrole may serve as a useful model compound for investigating the biological properties of safrole. A disadvantage in using 1′-acetoxysafrole in experiments with cultured cells is the short half-life of the compound in aqueous solution and the consequent necessity to use high concentrations. The maximum amount of repair synthesis in T98G cells is only about 15% of that obtained after saturating doses of UV, but quite similar to that following a saturating dose of activated aflatoxin B_1 in human diploid fibroblasts [20]. The classification of the repair patch size in DNA from 1′-acetoxysafrole-treated cells as "UV-like" (Fig. 5) places 1′-acetoxysafrole in a class similar to that of metabolically activated aflatoxin B_1 [20] and the furocoumarins, 8-methoxypsoralen and angelicin [21]. Although the 1′-acetoxysafrole repair patch size is determined to be about 80% of the patch size measured after UV, this may not

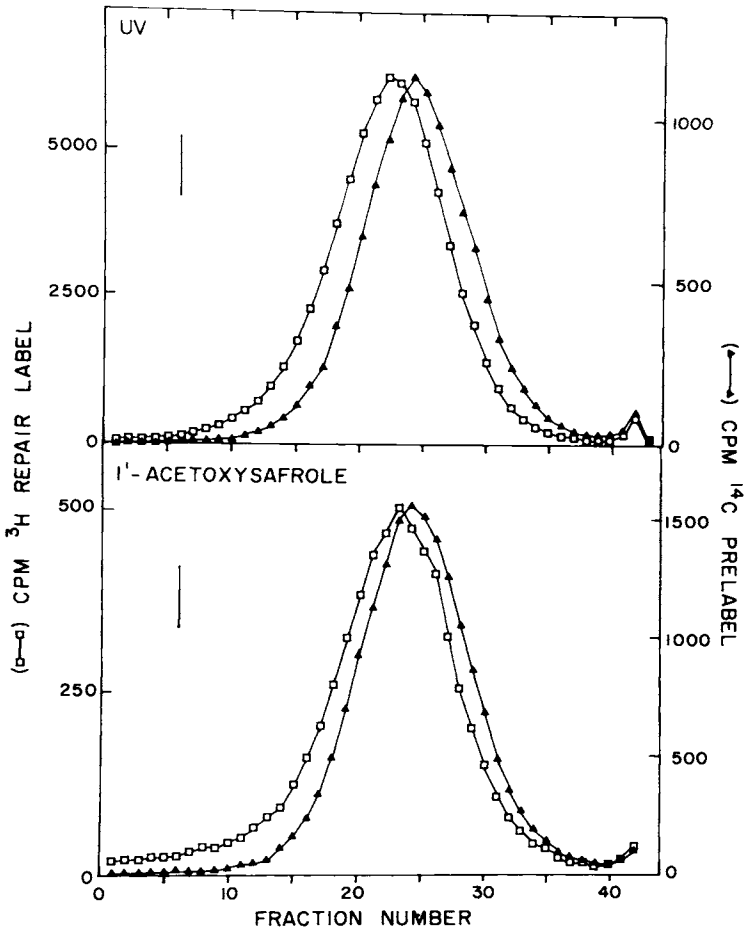

Fig. 5. Repair patch size distribution after exposure of T98G cells to UV or 1′acetoxysafrole. Cells were incubated in medium containing BrdUrd and (^{3}H)dThd, as described in Materials and Methods, and their parental density DNA was purified on neutral CsCl gradients, sonicated, and analyzed in the alkaline CsCl gradients shown. The tops of the gradients are to the right. Bars, position of 5-bromouracil-containing strand of unsonicated DNA.

reflect an actual difference in repair patch size. Our method assumes a uniform incorporation of BrdUrd into repaired regions of DNA at a frequency characteristic of the thymine content of human cellular DNA. However, UV predominantly dimerizes the thymine residues in DNA, with the result that the damaged regions may be relatively thymine-rich [22] and thus have an enriched frequency of BrdUrd residues incorporated by repair per unit length of DNA. 1′-Acetoxysafrole, on the other hand, reacts predominantly with guanine residues, so that damaged (and hence repaired) regions of DNA may be relatively guanine-rich and thymine-poor. In that case, the incorporation of density-labeled nucleotides per unit length of DNA would be reduced, and could then account for the apparently shorter repair patch size in 1′-acetoxysafrole-treated cells.

The application of the BrdUrd photolysis technique to determine DNA repair in 1′-acetoxysafrole-treated human cells has led to a much smaller estimate of the patch size than that obtained by our method [23]. The discrepancy may be related to the different levels of DNA damage introduced in the respective studies and/or to intrinsic features of the two methodologies [24]. Studies in progress are designed to correlate the DNA adducts formed from ^{3}H-labeled 1′-acetoxysafrole and their removal with measured levels of repair synthesis.

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