

Effects of Black Raspberry Extract and Protocatechuic Acid on Carcinogen-DNA Adducts and Mutagenesis, and Oxidative Stress in Rat and Human Oral Cells

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Abstract

Effects of black raspberry (BRB) extract and protocatechuic acid (PCA) on DNA adduct formation and mutagenesis induced by metabolites of dibenzo[a,l]pyrene (DBP) were investigated in rat oral fibroblasts. The DBP metabolites, (\pm)-anti-11,12-dihydroxy-11,12,-dihydrodibenzo[a,l]pyrene (DBP-diol) and 11,12-dihydroxy-13,14-epoxy-11,12,13,14-tetrahydrodibenzo[a,l]pyrene (DBPDE) induced dose-dependent DNA adducts and mutations. DBPDE was considerably more potent, whereas the parent compound had no significant effect. Treatment with BRB extract (BRBE) and PCA resulted in reduced DBP-derived DNA adduct levels and reduced mutagenesis induced by DBP-diol, but only BRBE was similarly effective against (DBPDE). BRBE did not directly inactivate DBPDE, but rather induced a cellular

response—enhanced DNA repair. When BRBE was added to cells 1 day after the DBP-diol, the BRBE greatly enhanced removal of DBP-derived DNA adducts. As oxidative stress can contribute to several stages of carcinogenesis, BRBE and PCA were investigated for their abilities to reduce oxidative stress in a human leukoplakia cell line by monitoring the redox indicator, 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF) in cellular and acellular systems. BRBE effectively inhibited the oxidation, but PCA was only minimally effective against H₂DCF. These results taken together provide evidence that BRBE and PCA can inhibit initiation of carcinogenesis by polycyclic aromatic hydrocarbons; and in addition, BRBE reduces oxidative stress. *Cancer Prev Res*; 9(8); 704–12. ©2016 AACR.

Introduction

Cancers of the oral cavity and pharynx comprise the sixth most common malignancies worldwide, representing a major international health problem (1). The 5-year survival rate for patients with head and neck squamous cell carcinoma (HNSCC) is only about 50% (2). In the United States, over 45,000 cases and about 11,000 deaths from HNSCC occur annually (3). Worldwide, the annual incidence of new cases exceeds 300,000 (1). The number of annual deaths from oral cancer is similar to that from melanoma (3). Patients with resected oral cancers have an increased incidence of second primary tumors

of the oral cavity (4, 5). Epidemiologic data provide strong support for exogenous factors such as tobacco, alcohol use, and human papillomavirus infection as being major causative agents (1, 3). In general, avoidance of risk factors has only been partially successful, as many individuals cannot change behavior—largely because smoking and alcohol have addictive effects.

As prevention is preferable to treatment, chemoprevention is a desirable approach. Numerous sources of phytochemicals have been proposed (6), and one that has shown promise in inhibiting head and neck cancers is freeze-dried black raspberry (BRB) (7). Many studies on black raspberries and their components have reported cancer preventive activity (8, 9). Topical treatment of dysplastic lesions in the oral cavity of humans with a 10% BRB gel, four times per day for 6 weeks, led to histologic regression of about 60% of the lesions; and several animal models have reported effectiveness of BRB against certain cancers (7–9). Although studies with whole BRB powder and with different BRB formulations are promising, there are multiple concerns regarding the "standardization" of whole berry powders. In view of this, Stoner's laboratory has conducted bio-fractionation studies of BRB powder to identify the bioactive constituents and has shown that the chemopreventive activity in multiple assays can be attributed significantly to the anthocyanins (AC), the most abundant class of compounds in BRBs, but they are expensive for routine chemoprevention. In addition, they are difficult to synthesize. Protocatechuic acid (PCA), which is commercially available and relatively inexpensive, accounts for about 70% of the

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metabolites of BRB ACs in humans (10), and is an inhibitor of chemically induced cancers in rodents when administered subsequent to the carcinogen (11).

Previously, we reported that the tobacco smoke carcinogen, dibenzo[a,l]pyrene (DBP; also known as Dibenzo[def,p]chrysene), and its ultimate carcinogenic metabolite, (\pm)-anti-11,12-dihydroxy-13,14-epoxy-11,12,13,14-tetrahydrodibenzo[a,l]pyrene (DBPDE), are mutagenic and carcinogenic in the mouse oral cavity (refs. 12, 13; see Fig. 1). We also reported that DBP and DBPDE form DNA adducts, preferentially with adenine (12, 13). Here, we report on the mutagenic effects and DNA adduct formation from the DBP metabolite, (\pm)-anti-11,12-dihydroxy-11,12,-dihydrodibenzo[a,l]pyrene (DBP-diol), and DBPDE in rat oral fibroblasts (OFB) containing a mutagenesis reporter gene (14). We also determined the effects of a BRBE and PCA, on mutagenesis and DNA adduct levels in OFB treated with DBP-diol and DBPDE. In addition, we report the effects of BRBE and PCA on oxidative stress, as assayed by their abilities to inhibit the oxidation of 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF) to its fluorescent product, DCF, in a precancerous human oral epithelial cell line.

Materials and Methods

Chemicals

DBP-diol and (\pm)-anti-DB[a,l]PDE were prepared according to a published method by our group (15). BRB powder and BRBE were prepared as described and provided by Dr. Stoner (11). The solvent used to obtain the BRBE from BRB powder was ethanol/H₂O (80:20). Using high-performance liquid chromatography (HPLC), the extract was shown to be composed of approximately 70% ACs (16). The remaining compounds have not been identified. H₂DCF was purchased from Biotium Inc. PCA was purchased from Thermo Sci. Acros Organics.

Cell lines

OFB and rat epithelial cell lines were derived from a *lacI* (BigBlue) Fisher 344 rat and were kindly provided by David Josephy (University of Guelph, Guelph, Canada). The preparation, characterization, and maintenance of these lines have been described (14). Cells were grown in DMEM/F12 medium (Corning cellgro) containing 5% FCS (Hyclone), Glutamine (0.29 mg/mL), and G418-sulfate and penicillin-streptomycin (all from Mediatech) at 0.22 mg/mL. Cells were passaged weekly.

MSK-Leuk1 cells were established from a premalignant leukoplakic lesion adjacent to a squamous cell carcinoma of the tongue (17, 18). The cells were obtained from Dr. Peter Sacks, who is emeritus faculty member in the same department as J.B. Guttenplan. The cells were authenticated by Genetica DNA Laboratories using short tandem repeat DNA profiling on December 8, 2015. Sequencing studies indicated that a GC>AT transition in exon 8 in one allele of p53, resulting in a glu to lys mutation in codon 286, was present in the MSK-Leuk1 cells (18). This cell line was routinely maintained in Keratinocyte Growth Medium (Lonza Bioresearch) grown to 70% confluence, and trypsinized with 0.125% trypsin-2 mmol/L EDTA solution before passage.

DNA adduct assays and mutagenesis studies

The OFB cell line was employed. Cells were grown in 1:1 DMEM/F12 (Corning cellgro) containing 5% FCS (Hyclone) to about 20% confluence and 50% for DNA adduct analysis. When

the cells reached the desired confluencies, the medium was replaced with 10 mL of DMEM/F12 medium without serum, and 2 hours later, the cells were treated with BRBE or PCA, and 4 hours later, DBP-diol or DBPDE was added to the desired concentrations. The concentrations of BRBE, PCA, DBP-diol, and DBPDE used are given in Figs. 1–6. One hour later, the serum concentration was brought to 5%. For mutagenesis, which requires replication, the cells were incubated at 37°C for 72 hours before harvesting. For DNA adduct measurements, cells were treated as above, but harvested only 24 hours after addition of DBP-diol or DBPDE, as replication was not necessary. Longer incubation periods would dilute DNA adducts with new DNA and allow more time for DNA repair.

Effect of time-after-treatment on DNA adduct levels

Three groups of OFB were treated with DBP-diol. One day later, DNA adducts were analyzed in one group, and BRBE was added to one of the remaining two groups of cells. One day subsequently (48 hours after addition of DBP-diol), DNA adducts were analyzed in both groups of cells.

Analysis of DNA adducts

As reported by us, DBPDE-dA is the major DNA adduct detected in mice treated with DBP (19). The method used for analysis of the DBPDE-dA adducts has been published (19–21). In brief, DNA was isolated from cells using the Qiagen DNA easy Kit as described by the manufacturer. Prior to enzymatic digestion, 150 pg of each [¹⁵N₅]-(-)-anti-trans- and [¹⁵N₅]-(-)-anti-cis-DBPDE-dA adducts were added to approximately 100 μ g DNA. DNA was hydrolyzed in the presence of 1 mol/L MgCl₂ (10 μ L/mg DNA) and DNase I (0.2 mg/mg DNA) at 37°C for 1.5 hours. Subsequently, nuclease P1 (20 μ g/mg DNA) snake venom phosphodiesterase (0.08 unit/mg DNA) and alkaline phosphatase (2 units/mg DNA) were used. An aliquot of the DNA hydrolysate was subjected to dA base analysis by HPLC. The remaining supernatant was partially purified by solid phase extraction using an Oasis HLB column (1 cm³, 30 mg; Waters Ltd.). Then, the analysis was carried out on an API 3200 LC/MS/MS triple quadrupole mass spectrometer interfaced with an Agilent 1200 series HPLC using an Agilent extend-c18 5 μ m 4.6 \times 150 mm column. Adducts were monitored in multiple reaction monitoring mode. The MS/MS transitions of *m/z* 604 \rightarrow *m/z* 335 and *m/z* 609 \rightarrow *m/z* 335 were monitored for targeted adducts and internal standards, respectively. Experiments using DBP-diol and DBPDE with and without BRBE or PCA were performed on different days, and the absolute values of DNA adduct levels without inhibitor varied from experiment to experiment, but were internally consistent. Each condition was assayed from triplicate plates except where there are no error bars; these represent single plates and were used to establish a dose range. Results in the figures represent the major adduct, (-) anti-trans-DBP-dA.

Mutagenesis assay

After treatment of the cells, with mutagen in the presence or absence of BRBE or PCA DNA was extracted using a Recoverase kit (Agilent Technologies) as per the manufacturer's instructions, which involve isolation of nuclei, cell lysis, digestion with protease K, RNase, and dialysis on a membrane. Phage packaging was carried out using a phage packaging mix prepared from bacterial strains *E. coli* NM759 and BHB2688, generously supplied by Dr. Peter Glazer (Yale, University

School of Medicine, New Haven, CT) according to published methods (22).

The *cII* mutagenesis assay was then employed (23). Briefly, the isolated DNA was treated with the phage packaging extract, which contains all the components necessary for the *in vitro* assembly of a lambda phage containing in the phage head a vector that includes the bacterial *lacI* locus and the *cII* gene, the target for the mutagenesis assay (23–27). In appropriate *E. coli* (*E. coli* 1250) host cells, under specified conditions (25°C), only mutants give rise to phage plaques, whereas at 37°C, all infected cells give rise to plaques, providing a phage titer (23–27). The mutant fraction (MF) is the ratio of mutant to nonmutant plaques. Plates for each condition were done in triplicate. Experiments using DBP-diol and DBPDE with and without BRBE or PCA were performed on different days, and the absolute values for mutagenesis with and without inhibitor vary from experiment to experiment, but are internally consistent.

Oxidative stress

The oxidative stress assays are dependent on the oxidation of H₂DCF to DCF—the latter compound being much more fluorescent than the reduced form. In the cellular assays, 5,000 to 10,000 MSK-Leuk1 cells were plated in 96-well plates in keratinocyte growth medium, and the next day, BRBE or PCA was added at the concentrations mentioned above. Four hours later, H₂DCF was added to a concentration of 5 μmol/L. Fluorescence was read in a Molecular Devices SpectraMax-M5 plate reader using 495 nm excitation and 525 nm emission wavelengths at 30-minute intervals up to 90 minutes. For the acellular assay, 0.1 mmol/L hydrogen peroxide, with and without BRBE or PCA in KGM, was incubated for 4 hours at 37°C. H₂DCF was then added and fluorescence was read at 60 minutes. The background fluorescence of cells alone was about 3% of the final reading and was not subtracted. For the acellular assay without hydrogen peroxide, DCF with or without BRBE or PCA was incubated overnight with shaking at 37°C, and fluorescence was read 16 hours after the start of the incubation. H₂DCF in PBS or KGM without cells was essentially nonfluorescent after 90 minutes. BRBE and PCA at the concentrations employed did not affect the fluorescence of DCF, and were not significantly fluorescent at the concentrations employed.

Analysis of DBP-tetrols

DBP tetrols were analyzed by an HPLC method previously described by us (18, 28). Elution was performed using a Shimadzu LC20AD system and a Waters C18 Symmetry column (2.1 × 150 mm, 3.5 micron particle size) at a flow rate of 0.17 mL/min in a pH 4.0, 10 mmol/L ammonium phosphate buffer containing 45% acetonitrile. A fluorescence detector (Shimadzu; RF10Axl) was set at 344 nm excitation and 400 emission wavelengths.

Results

Dose response for DNA adducts resulting from treatment with DBP, DBP-diol, and DBPDE

In order to determine an appropriate dose range for experiments using DBP, DBP-diol, and DBPDE, DNA adducts produced in OFB and epithelial cells by these compounds were measured. No measurable adducts were produced by DBP. The major DNA adducts produced by both DBP-dihydrodiol and DBPDE were the *cis* and *trans* anti-DBPDE-dA (Fig. 2A–D).

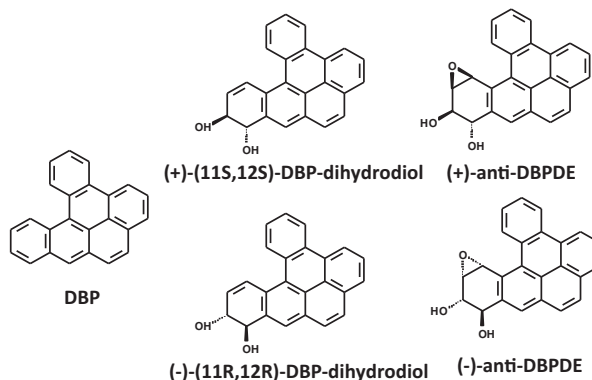


Figure 1.

Structures of DBP, DBP-diol, and two isomers of anti-DBPDE.

In Fig. 2D, peaks with retention time (RT) 26.5 and 29 minutes are DBPDE-dG adducts; peaks with RT 30.5 and 36.4 are (+)-DBPDE-dA adducts. They are all correlated with the doses of DBPDE, but we only quantified (-)-DBPDE-dA adducts because they are more important to DBP-induced carcinogenesis as reported in our previous publications (21, 29). DBPDE was much more effective at producing adducts than DBP-diol. The dose–response results were then used to determine concentrations of DBP-diol and DBPDE for subsequent experiments.

Mutagenesis induced by DBP, DBP-diol, and DBPDE

Mutagenesis induced by DBP, DBP-diol, and DBPDE was monitored in the *lacI* rat oral epithelial and fibroblast cells. DBP was at best very weakly mutagenic (results not shown) and was not studied further. Preliminary studies revealed that DBP-diol and DBPDE were similarly mutagenic in both cell lines, but background mutation levels were greater in the epithelial cell line, resulting in the fibroblast line being more sensitive to mutagenesis. Therefore, the fibroblast cell line was used for further experiments. Results of the mutagenesis assay are shown in Fig. 3A and B. DBPDE was much more potent than DBP-diol. The relative potencies were DBPDE, 9,600; DBP-diol, 14.7; DBP, 1.

Effects of BRBE and PCA on DNA adduct formation by DBP-diol and DBPDE

BRBE produced a significant inhibition (about 50%) of DBP-diol-induced DNA adduct formation at 66 μg/mL medium and greater inhibition at higher doses (Fig. 4A). It also inhibited DNA adduct formation by DBPDE; similar to the effects on adduct formation by DBP-diol, it was very effective at concentrations of 75 μg/mL and above (Fig. 4B). PCA also inhibited DNA adduct formation by DBP-diol (Fig. 4A), but in contrast to BRBE, it had no effect on adduct formation by DBPDE at the concentrations employed (Fig. 4C).

Effect of BRBE on removal of DBP-diol-induced DNA adducts

When BRBE was added to cells 1 day after the DBP-diol, it greatly enhanced removal of DBP-derived DNA adducts over the next 24 hours (Fig. 4D). One day after treatment with 1.5 μmol/L DBP-diol, levels of the DBP-dA adduct were about 16×10^{-6} adducts/dA. One day later, they declined to

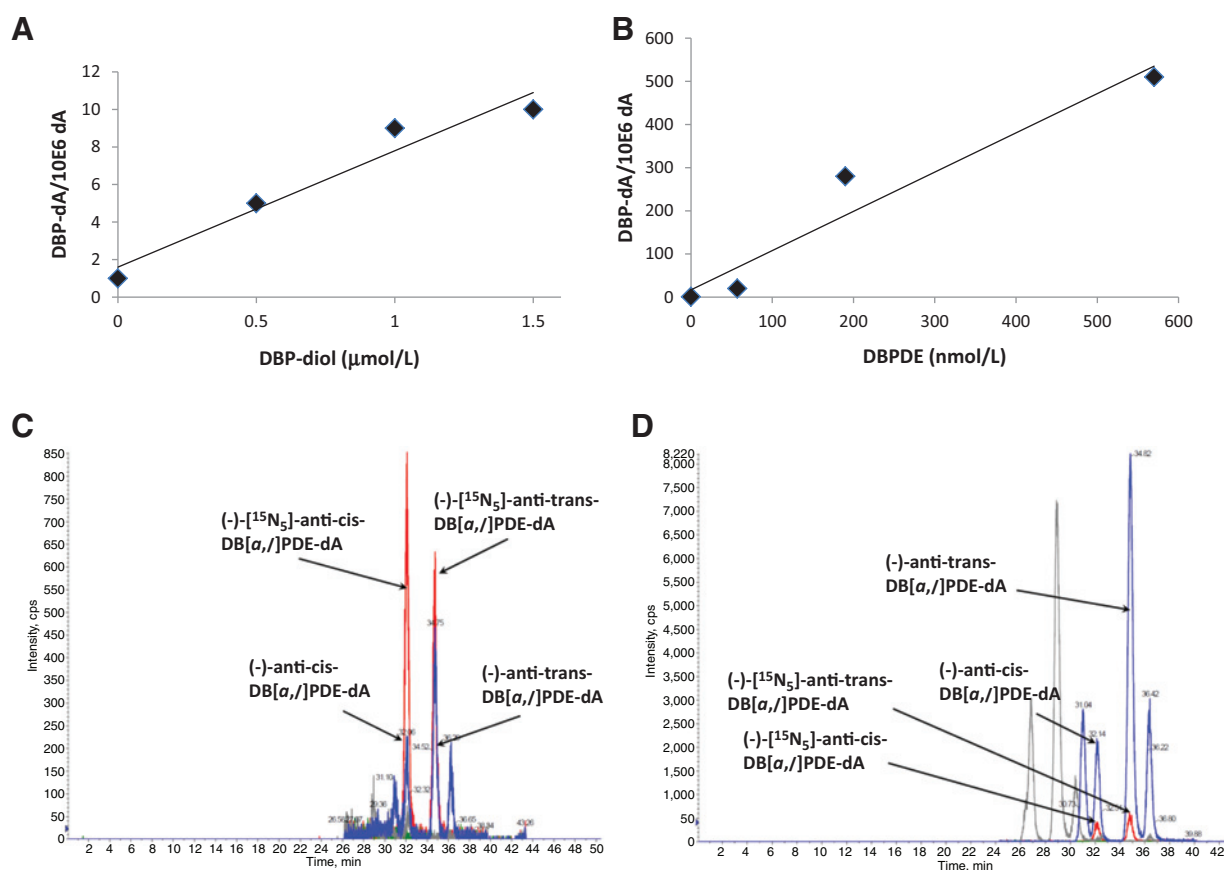


Figure 2.

Dose response for DNA adducts. **A**, dependence of DNA adduct level on concentration of DBP-diol in OFB. **B**, dependence of DNA adduct level on concentration of DBPDE in OFB. **C**, a representative HPLC-MS/MS chromatogram of DBP-diol-induced dA adducts. **D**, a representative HPLC-MS/MS chromatogram of DBPDE-induced dA adducts. The values in **A** and **B** represent DNA adducts from single plates and were used to establish a dose range.

$8.1 \text{ adducts} \times 10^{-6} \text{ adducts/dA}$. However, when the OFB were treated with $75 \mu\text{g/mL}$ BRBE 1 day after DBP-diol treatment, the adduct level declined to $2.9 \text{ adducts} \times 10^{-6} \text{ adducts/dA}$, a major enhancement in removal.

Effects of BRBE and PCA on mutagenesis by DBP-diol and DBPDE

BRBE was an effective inhibitor of mutagenesis induced by DBP-diol at the concentrations employed (Fig. 5A). The inhibition was dose-responsive with slightly above 50% inhibition at $25 \mu\text{g/mL}$. BRBE was also effective at reducing mutagenesis induced by DBPDE (Fig. 5C). PCA was moderately effective at inhibiting mutagenesis induced by DBP-diol, showing a dose-response, although the inhibition only reached significance at $1 \mu\text{mol/L}$ and above (Fig. 5B). PCA had no noticeable effect on mutagenesis induced by DBPDE at the concentrations employed (Fig. 5D).

Investigation of possible direct effects of BRBE on DBPDE

In contrast to DBP-diol, which must be further metabolized to exert its mutagenic and carcinogenic effects, fjord-region diol-epoxides like DBPDE are short-lived highly reactive electrophiles that bind to DNA without further metabolism (30). Because BRBE inhibited DNA adduct formation by DBPDE, we considered the

possibility that there was a direct reaction between the extract and DBPDE. If some component (s) of BRBE reacted with DBPDE before the DBPDE could permeate the cells or when DBPDE was within the cells, this could explain the mechanism of the observed inhibitory effects. As DBP-diol is metabolized to DBPDE, this mechanism could also apply to inhibition of DNA adduct formation and mutagenesis by DBP-diol. To test this possibility, we investigated whether the products from the spontaneous decomposition of the DBPDE were affected by the presence of BRBE. Upon spontaneous hydrolysis of DBPDE in aqueous medium, two products are formed—the cis and trans (-) anti-11,12,13,14-tetrahydro-11,12,13,14-tetrahydroxy-DBP (DBP-tetrols). DBPDE was incubated in keratinocyte growth medium for 30 minutes, 1 hour, 2 hours, and overnight at 25°C . After incubation, the solutions were applied to an HPLC, and the two expected peaks were observed (Fig. 1; Supplementary Data). The concentrations of the products increased from 30 to 60 minutes, but did not change after that—even after an overnight incubation. A second solution containing the same concentration of DBPDE plus $50 \mu\text{g/mL}$ BRBE, and incubated in parallel, resulted in the same two products at the same final concentrations, indicating that BRB components do not react with DBPDE and prevent it from reacting with DNA and other molecules.

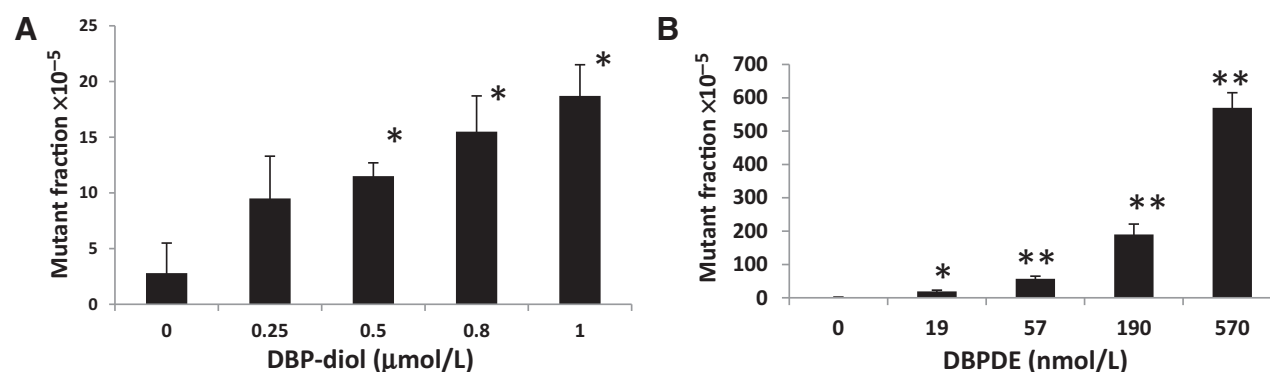


Figure 3.

A, dependence of mutagenesis on concentration of DBP-diol in OFB. **B**, dependence of mutagenesis on concentration of DBPDE in OFB. *, $P < 0.05$; **, $P < 0.01$ in a one-tailed t test versus control (no DBP metabolite).

Effects of BRBE and PCA on oxidative stress

As oxidative stress can play a role in initiation of carcinogenesis, as well as during the promotion and progression stages (31, 32), we investigated whether BRBE and PCA could inhibit oxidative stress. To determine whether BRBE affects intracellular oxidative stress, we monitored the ability of the precancerous human oral epithelial cell line, MSK Leuk1, to oxidize H₂DCF to DCF in the presence and absence of BRBE. The oxidized form (DCF) is highly fluorescent, in contrast to the reduced form (H₂DCF). BRBE was very effective at reducing the

rate of oxidation. Even at a relatively low dose (2.5 μg/mL), it reduced the rate of oxidation by over 50%, and the effect was even more pronounced at higher concentrations (Fig. 6A). In contrast, PCA exhibited only a minor effect on oxidative stress, with a statistically significant inhibition reached at the relatively high dose of 4 μmol/L (Fig. 6B).

We also tested whether BRBE could inhibit the oxidation of H₂DCF extracellularly. We preincubated H₂DCF with 1 mmol/L hydrogen peroxide in the presence and absence of BRBE for 3 hours in PBS before addition of DCF. BRBE reduced the rate

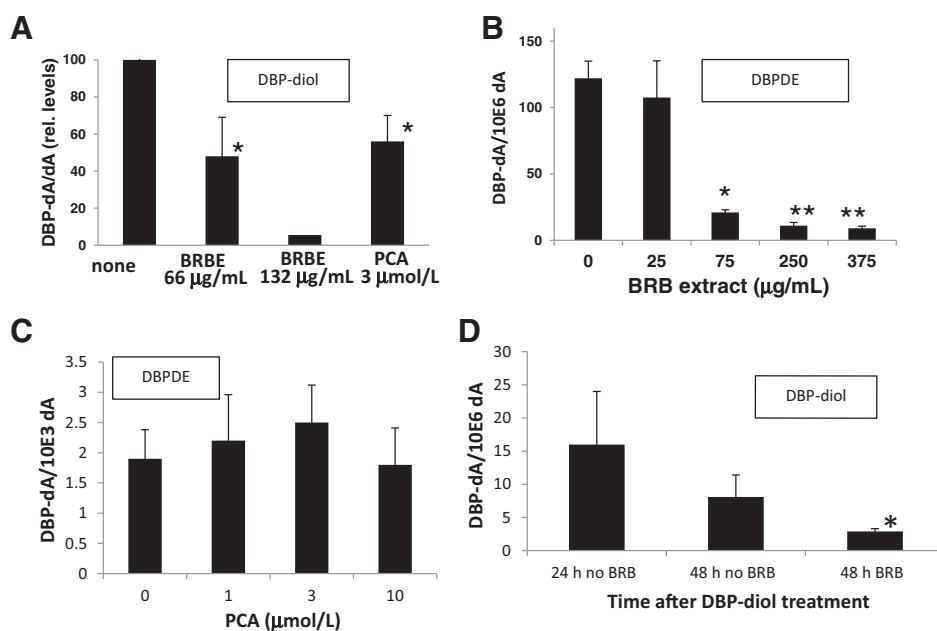
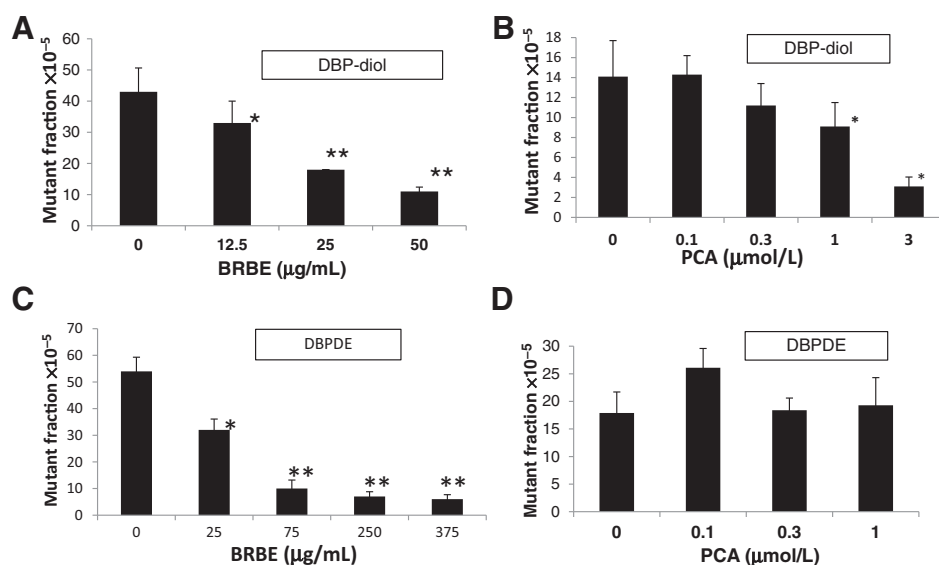


Figure 4.

A, effects of BRBE and PCA on DNA adducts produced by DBP-diol in OFB. DBP-diol, 0.5 μmol/L. **B**, effect of BRBE on DNA adducts produced by DBPDE in OFB. DBPDE, 70 nmol/L. For the highest dose of DBPDE, there was only one sample that yielded sufficient DNA for adduct analysis; the single result is plotted in the figure. **C**, effect of PCA on DNA adducts produced by DBPDE in OFB. DBPDE, 140 nmol/L. *, $P < 0.05$; **, $P < 0.01$ in a one-tailed t test versus control (no DBP metabolite). Experiments using BRBE and PCA in the presence of DBP-diol were performed on different days and are expressed as a percentage of the control (DBP-diol-alone). **D**, effect of BRBE on removal of DBPDE-dA adducts. OFB were treated with 1.5 μmol/L DBP-diol. One day later, DNA adducts were analyzed in one group, and 75 μg/mL BRBE was added to a second group of cells, whereas a third group of DBP-diol-treated cells was incubated without further additions. One day later (48 hours after addition of DBP-diol), adducts were analyzed in DBP-diol-treated OFB, with and without BRBE. *, $P < 0.05$ in a Mann-Whitney U test, and 0.055 in a t test with unequal variances versus 48-hour group with no BRBE.

Figure 5.

A, effect of BRBE on mutagenesis induced by DBP-diol in OFB, DBP-diol, 0.5 $\mu\text{mol/L}$. **B**, effect of PCA on mutagenesis induced by DBP-diol in OFB, DBP-diol, 0.5 $\mu\text{mol/L}$. **C**, effect of BRBE on mutagenesis induced by DBP-diol in OFB, DBPDE, 70 nmol/L. **D**, effect of PCA on mutagenesis induced by DBPDE in OFB, DBPDE, 70 nmol/L. *, $P < 0.05$; **, $P < 0.01$ in a one-tailed t test versus control (no DBP metabolite). Mutagenesis experiments depicted in each panel in Figs. 3 and 5 were all performed on different days, so the absolute MF values for a particular mutagen may differ from day to day, but results are internally consistent.



of oxidation by about 50% at 5 $\mu\text{g/mL}$, indicating it need not generate an intracellular response to act as an antioxidant (Fig. 6C). This experiment demonstrated that a component of BRBE reacted with preformed hydrogen peroxide. We also investigated whether BRBE could inhibit the ambient atmospheric oxidation of H_2DCF . H_2DCF is oxidized to DCF in aqueous solution, but less rapidly than in the presence of hydrogen peroxide or when taken up by the MSK cells. We incubated H_2DCF with and without BRBE, in the absence of any added oxidant for 16 hours at 37°C and then measured fluorescence. Similar to the results with hydrogen peroxide, BRBE inhibited the oxidation of H_2DCF by about 50% at a concentration of 5 $\mu\text{g/mL}$ (Fig. 6D).

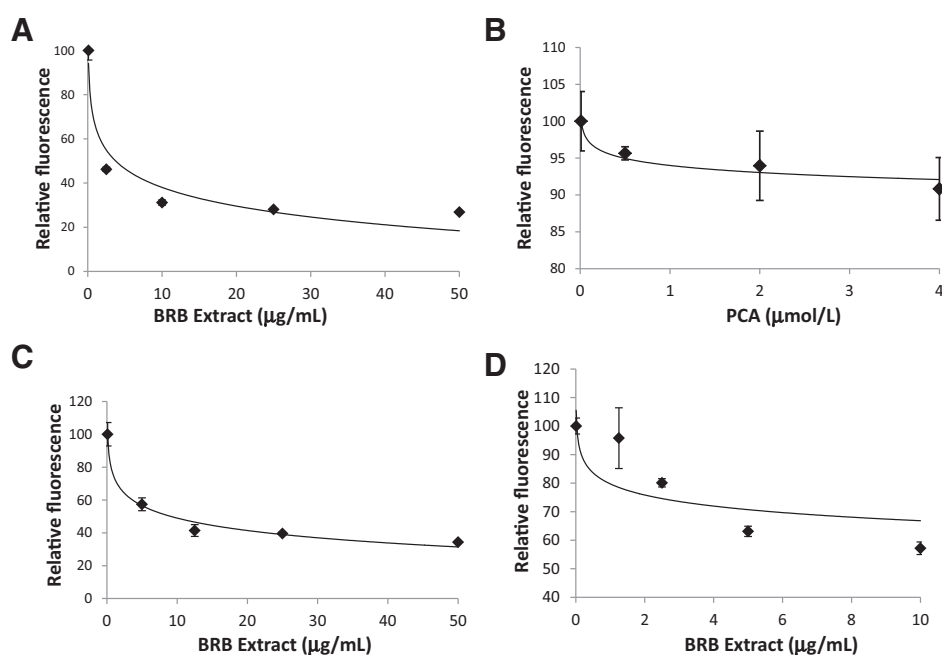
Discussion

BRB powder and the ethanol/ H_2O -soluble (80:20) extract have been effective at inhibiting carcinogenesis in a number of *in vitro* and *in vivo* experimental systems (7, 9, 11). Exposure to BRB results in a reduced rate of growth of precancerous cells, reduced inflammation, oxidative stress and angiogenesis, increased apoptosis, and a reduced tumor multiplicity in initiated animals. In those studies, BRB is functioning to inhibit the promotion and progression stages of carcinogenesis. Here, we investigated the effects of an ethanol/ H_2O soluble (80:20) extract of BRB and a major *in vivo* metabolite, PCA, on processes important for initiation of carcinogenesis—DNA damage, mutagenesis, and potentially, oxidative stress. In experimental animals, DBP is metabolized to (among other metabolites) DBP-epoxide, then to DBP-diol, and finally to DBPDE (20). The diol is thought to represent the proximate mutagenic and carcinogenic metabolite of DBP, and DBPDE is the ultimate mutagen and carcinogen (20). We have previously reported that in mice, DBP and DBPDE form DNA adducts in oral tissue—predominantly with adenine (21) and a much higher percentage of the mutations were found at A:T base pairs than in untreated mice (12, 13). Significant fractions of mutations at G:C pairs were also observed.

Consistent with the above metabolic pathway for DBP, DBPDE was much more potent than DBP-diol, and DBP-diol was more potent than DBP in the induction of DNA adducts and mutations. Because the OFB were unable to metabolize

sufficient DBP to produce detectable levels of DNA adducts or lead to mutagenesis, further experiments on inhibition of DNA adduct formation and mutagenesis were carried out using DBP-diol and DBPDE. We then investigated inhibition of these processes by the BRBE and PCA. BRB contains about 4% to 5% by dry weight of ACs, and they are thought to be responsible for many of the reported effects of BRB on carcinogenesis and inflammation (11, 16). PCA is a major metabolite of ACs and is produced by microbial metabolism of ACs in the gut (11, 33). There is evidence that some inhibitory effects of BRB and ACs on carcinogenesis can be attributed to PCA (11).

BRBs may act on different stages of carcinogenesis, and in a number of experimental models of carcinogenesis, BRB and ACs were shown to inhibit growth of implanted tumor cells or retard growth of tumors in animals predisposed for cancer (34). In some cases, they were administered in the diet after treatment with a carcinogen (11). In such models, their effects were on promotion and progression of carcinogenesis. There have been studies where they were administered before, during, and after treatment with a carcinogen, but in those cases, it was not possible to determine at which stage of carcinogenesis inhibition was occurring (34). Here, we investigated effects of BRB on processes involved in tumor initiation, using an *in vitro* system, in which formation of DNA adducts by a proximate and ultimate carcinogen was measured, and mutagenesis (a consequence of DNA damage) was also monitored. In addition, effects of BRBE on oxidative stress were examined, as oxidative stress contributes to several stages of carcinogenesis. Pretreatment of cells with BRBE followed by treatment with DBP-diol led to a reduction in DBPDE-induced DNA adducts and mutagenesis, compared with cells treated with DBP-diol alone. Presumably, the reduction in adducts was responsible for the reduction in mutagenesis. Potential mechanisms of inhibition of initiating effects of DBP-diol are (i) direct inhibition of its metabolizing to DBPDE or altering its metabolism so that less DBPDE is produced, and/or (ii) trapping or detoxifying DBPDE before it reacts with DNA. BRBE also inhibited mutagenesis and DNA adduct formation by DBPDE. In contrast to DBP-diol, DBPDE is the ultimate mutagenic metabolite of DBP-diol, and inhibition of the reaction of DBPDE with DNA cannot be attributed to inhibition of its metabolism to a more

**Figure 6.**

A, effect of BRBE on fluorescence intensity of DCF in human oral epithelial cells. **B**, effect of PCA acid on fluorescence intensity of DCF in human oral epithelial cells. **C**, effect of BRBE on fluorescence intensity of DCF in the presence of hydrogen peroxide in the absence of cells. **D**, effect of BRBE on fluorescence intensity of DCF in the absence of cells when incubated overnight at 37°C. Results are expressed as relative fluorescence intensity, where the control (no BRBE) is 100%. The trend lines are logarithmic. For all points with BRBE, $P < 0.01$ in a one-tailed t test versus control (no BRBE) except the 2 µg/mL point in **D**, which was not significantly different than control. There was no statistically significant effect of PCA on fluorescence intensity at any of the concentrations tested.

potent mutagen. The inhibitory effects of BRBE on adduct formation by DBPDE likely result from trapping or enzymatic detoxification of DBPDE.

To investigate the possibility that BRBE can trap DBPDE and prevent it from reacting with nucleophiles (including DNA), we incubated DBPDE in culture medium in the presence and absence of 50 µg/mL BRBE. DBPDE spontaneously decomposed to form two DBP-tetrols. We allowed the incubation proceed to completion and then measured the concentrations of the two DBP-tetrols. The concentration of tetrols produced was unchanged by the presence of BRBE in the incubation solution. If a component of the BRBE reacted with DBPDE before it could hydrolyze, then the concentration of the tetrols would have been reduced. This was not the case, and we conclude that the inhibitory effects of BRB on DNA adduct formation and mutagenesis likely result from a cellular response to DBPDE. One possibility is that ACs induce the expression of NAD(P)H dehydrogenase [quinone] (NQO1) which is a sentinel for induction of phase II enzymes (35). Several of these enzymes, such as the glutathione transferases, may conjugate DBPDE and thus reduce its concentration. It has already been reported that several glutathione transferases are capable of catalyzing the conjugation of DBPDE with glutathione (36, 37). We also show here that BRBE enhances the removal of DBPDE-DNA adducts. The mechanism for inhibition by BRB of DBP-diol-induced adduct formation and mutagenesis may be the same as that for its inhibition of DBPDE, as DBP-diol is metabolized to DBPDE. However, the conversion of DBP-diol to DBPDE may also be subject to enzyme inhibition.

We also investigated the effects of PCA, a major metabolite of ACs, on DNA damage and mutagenesis by DBP-diol and DBPDE. Pretreatment with PCA also led to inhibition of DNA adduct formation and mutagenesis by DBP-diol, but not by DBPDE. Hence, the reason for its inhibitory effects likely involves its inhibition of the formation of DBPDE, either by enzyme inhibition or induction of phase II enzymes. PCA is readily available and would represent a convenient substitute for BRBE; the latter must be produced by more involved processing.

An important consideration is whether the levels of inhibitors employed here are relevant to *in vivo* levels. Plasma levels of PCA in volunteers consuming berry powder were 1 to 2 orders of magnitude below those used here (38). However in mice fed PCA in food, higher plasma levels (38) were achieved. In principle then, blood levels of PCA similar to those used here in cell culture could be achievable by use of supplemental dietary PCA.

For ACs, it has been reported that intraoral delivery of a 10% (w/w) bioadhesive freeze-dried BRB gel to human subjects led to a mean oral tissue concentration of about 0.5 µg AC/mg protein 5 minutes after administration of the gel (39), although there were wide interindividual variations. As typical tissue concentrations of proteins are about 15%, an average tissue concentration would be 75 µg/mL (150 mg/g tissue \times 0.5 µg AC/mg = 75 µg AC/g tissue). This is only for a single time point, but suggests the concentrations employed here are consistent with levels delivered to humans. Also, levels in saliva are in the same range as employed here, but again with very wide interindividual variations (39). The results reported here, taken together with the previous report, suggest that topical application of an BRBE or AC-enriched gel could inhibit DNA damage and mutagenesis induced by polycyclic aromatic hydrocarbon (PAH) in the human oral cavity.

BRBE was an effective inhibitor of cellular oxidative stress as measured by its ability to reduce the rate of oxidation of H₂DCF to DCF. Significant inhibition was observed at concentrations well below those necessary to inhibit DNA adduct formation. It has been reported that BRB can induce the antioxidant response element in liver cells, resulting in increased levels of antioxidant enzymes and glutathione (40). Also, grape seed ACs induce phase II enzymes in MCF10A breast cells and inhibit H₂DCF oxidation (41). These reports suggest that cellular responses to AC treatment can lead to the antioxidant effect of BRB. However, we observed that BRB inhibited the oxidation of DCF by hydrogen peroxide or molecular oxygen in the absence of cells. Our results are consistent with either inhibition resulting from a cellular response to BRBE

or direct reduction of intracellular oxidants by BRBE. The results thus far do not allow us to choose between the two possibilities, or a combination of the two.

In conclusion, BRBE at concentrations leading to AC levels achievable by topical application of BRB led to inhibition of DNA adduct formation resulting from the DBP metabolites, DBP-diol, and DBPDE in a rat oral cell model. Similar inhibition of mutagenesis induced by DBP-diol and DBPDE was also observed. PCA was an inhibitor of DNA adduct formation and mutagenesis induced by DBP-diol, but not DBPDE. BRBE but not PCA reduced oxidative stress. BRB is potentially an inhibitor of carcinogenesis initiation by tobacco smoke PAH, and its antioxidant ability may play a role in inhibiting several steps in multistage carcinogenesis.

Disclosure of Potential Conflicts of Interest

G.D. Stoner has ownership interest (including patents) in BerriProducts, LLC. No potential conflicts of interest were disclosed by the other authors.

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