Induction of Glutathione Transferases and NAD(P)H:Quinone Reductase by Fumaric Acid Derivatives in Rodent Cells and Tissues

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ABSTRACT

Dimethyl fumarate and dimethyl maleate are potent inducers of cyto- soluble NAD(P)H:quinone (acceptor) oxidoreductase (here designated quinone reductase) activity in Hepa 1c1c7 murine hepatoma cells in culture, whereas fumaric and maleic acids are much less potent, in agreement with the much greater reactivity of the esters as Michael reaction acceptors (P. Talalay, M. J. De Long, and H. J. Prochaska, Proc. Natl. Acad. Sci. USA, 85: 8261-8265, 1988). Dimethyl fumarate also induced quinone reductase in mutants of the Hepa 1c1c7 cell line that were either defective in the Ah receptor or in cytochrome P-450 activity, thereby establishing that this compound is a monofunctional inducer (H. J. Prochaska and P. Talalay, Cancer Res., 48: 4776-4782, 1988). Addition of dimethyl fumarate to the diet of female CD-1 mice and female Sprague-Dawley rats at 0.2-0.5% concentrations elevated cytosolic glutathione transferases and quinone reductase activities in a variety of organs, whereas much higher concentrations of fumaric acid were only marginally active. The widespread induction of such detoxication enzymes by dimethyl fumarate suggests the potential value of this compound as a protective agent against chemical carcinogenesis and other forms of electrophile toxicity. This proposal is supported by the finding that the concentrations of dimethyl fumarate required to obtain substantial enzyme inductions were well tolerated by rodents. Furthermore, the parent fumaric acid has low chronic toxicity and is a naturally occurring metabolic intermediate that is already in the food chain as an additive, and fumarate salts and esters are used for therapeutic purposes in man.

INTRODUCTION

A variety of structurally dissimilar chemical agents block the neoplastic and toxic effects of chemical carcinogens, a process that has been designated chemoprotection (1, 2). Many chemoprotectors induce enzymes of xenobiotic metabolism in cells in culture and in the liver and peripheral organs of mice and rats (2). Such inducers are of two types (3): bifunctional and monofunctional. Bifunctional inducers elevate the activities of both Phase 1 enzymes (such as cytochrome P-450), which play a central role in activating carcinogens, and Phase 2 enzymes (such as glutathione transferases (EC 2.5.1.18) and NAD(P)H:quinone oxidoreductase (EC 1.6.99.2)), which are largely involved in detoxication reactions. In contrast, monofunctional inducers elevate the activities of Phase 2 enzymes selectively. Whereas the mechanism of cytochrome P-450 induc- tion by bifunctional inducers (polycyclic aromatics, azo dyes, tetrachlorodibenzo-p-dioxin) involves the binding of these inducers to a complementary cytosolic Ah (Aryl Hydrocarbon) receptor (6-8), the inductive mechanism of Phase 2 enzymes by monofunctional inducers depends on the presence or acquisition by metabolism of electrophilic centers (9, 10). Thus, some monofunctional inducers are conventional Michael reaction acceptors, whereas others are subject to nucleophilic displacement or addition reactions. The electrophilic induction signal was identified by measuring QR* in Hepa 1c1c7 murine hepatoma cells in culture (9, 11, 12). With the help of this system several new classes of monofunctional inducers were characterized, including α,β-unsaturated lactones and carboxylic acid derivatives, e.g., coumarins, fumarates, maleates, acrylates, cinnamates, and related compounds.

In developing chemoprotective enzyme inducers for use in man, attention must be accorded to three desirable characteristics of such agents: (a) capacity for monofunctional rather than bifunctional induction. This minimizes the potential complications of carcinogen activation through the induction of certain cytochromes P-450; (b) ability to raise Phase 2 enzyme levels in multiple tissues, thereby enhancing the potential for protecting a variety of organs; (c) low toxicity, e.g., presence in the food chain or in living matter, thereby minimizing the need for extensive, long-term toxicity testing prior to clinical trials. Based on these desirable characteristics, we have selected from among the many known inducers of Phase 2 enzymes (9) a fumaric acid derivative, DIMEFU, for detailed study in cell culture and in rodents. Fumarate (the parent compound) is one of the few monofunctional inducers that is an endogenous constituent and an essential metabolic intermediate of animal tissues. Fumarate has low toxicity, is used widely as a food additive, and is on the “Generally Recognized As Safe,” list. Chronic toxicological studies in several species have found fumarate to be free of significant toxicity (13-15). Furthermore, oral ferrous fumarate is used widely to treat iron deficiency in man, and alkyl esters of fumaric acid (specifically DIMEFU) have been used successfully both topically and systemically in the treatment of psoriasis in man (see Ref. 16 and references therein). Moreover, administration of fumaric acid to rodents protects against chemical carcinogenesis (17, 18). We show in this paper that DIMEFU has many of the desirable characteristics of a useful chemoprotective agent and deserves further exploration as a protector against carcinogenesis and other forms of electrophile toxicity.

MATERIALS AND METHODS

Materials. Esters of fumarate, maleate, and succinate were obtained from Aldrich Chemical Co. (Milwaukee, WI). Crystalline dimethyl...
fumarate was ground to a fine powder in a mortar prior to addition to the diets. The powdered AIN 76A semipurified diet was from ICN Biomedicals (Costa Mesa, CA), and the powdered Purina 5001 diet was from Purina Ralston (St. Louis, MO). BHA and ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethyloxolinoine) were from Sigma Chemical Co. (St. Louis, MO).

Measurement of Quinone Reductase in Hepa 1c1c7 Murine Hepatoma Cells. The method for measuring the potency of inducers in raising QR specific activities in these cells has been described (12). For the present experiments, Hepa 1c1c7 cells were grown in 96-well microtiter plates and QR was measured on cell lysates (19). This procedure was modified by use in the medium of fetal calf serum that had been heated for 90 min at 55°C in the presence of activated charcoal (1 g/100 ml; Sigma), and filtered twice through 0.22-µm filters. This treatment increased the induction ratios of QR by lowering the basal activities of the enzyme, and by removing inhibitor(s) of induction present in untreated serum. The Hepa 1c1c7 cells were plated at 10,000 cells/microtiter plate well (150 µl of medium/well) and grown for 24 h. Serial dilutions of inducers were added in 0.1% (by volume) of acetonitrile in fresh medium, and the QR specific activities were determined after a 48-h exposure to the inducers. A plot of the ratio of QR specific activities of treated cells to control cells (which had received the solvent only) as a function of inducer concentration (Fig. 1) permitted the determination of the CD (Concentration required to Double the specific activity). All sets of determinations included plates induced with a series of concentrations (0.01-1.0 µM) of β-naphthoflavone as a standard inducer. The mean CD value for β-naphthoflavone was 35 nm.

Two mutants of Hepa 1c1c7 cells were also used. The c1 mutant (a gift from O. Hankinson, University of California, Los Angeles, CA) synthesizes an inactive (truncated) cytochrome P-450 gene product (20), whereas the BP∥cl mutant (a gift from James P. Whitlock, Jr., Stanford University, Palo Alto, CA) contains no detectable aryl hydrocarbon hydroxylase activity or its mRNA. It has a normal Ah receptor, but does not translocate the ligand-bound receptor to the nucleus (21). The mutants of Hepa 1c1c7 cells were grown and induced under similar conditions to the wild-type cell line (12, 22).

Treatment of Animals and Collection of Tissues. Weanling female Sprague-Dawley rats were fed a powdered AIN 76A diet supplemented with 0.2, 0.4, or 0.5% (by weight) of DIMEFU for 14 days beginning at 25 days of age. Control groups were fed this diet without DIMEFU. Treatment of Animals and Collection of Tissues. Weanling female Sprague-Dawley rats were fed a powdered AIN 76A diet supplemented with 0.2, 0.4, or 0.5% (by weight) of DIMEFU for 14 days beginning at 25 days of age. Control groups were fed this diet without DIMEFU. A positive control group received 0.4% (by weight) ethoxyquin in the diet. The rats were housed in plastic cages and were weighed daily during the feeding of the experimental diets.

Female CD-1 mice (Charles River Laboratories, Wilmington, MA) were fed a powdered Purina 5001 laboratory chow, supplemented with 0.2 or 0.5% (by weight) of DIMEFU for 14 days beginning at 45 days of age. A positive control group received a diet containing 0.75% 2(3)- tert-butyl-4-hydroxyanisole. The mice were housed in hanging cages without bedding, and were weighed daily during the feeding of the experimental diets.

The animals were killed by CO₂ inhalation and the tissues were removed, frozen in liquid nitrogen, and stored at −80°C until they were processed for enzyme assays. The mouse livers were perfused via the portal vein with cold 0.15 M KCl containing 2 mM EDTA, pH 7.5, prior to freezing. The rat livers were not perfused. All animal experiments were in compliance with “Guide for the Care and Use of Laboratory Animals,” NIH Publication 81-2385, United States Department of Health and Human Services, Washington, DC.

Preparation of Tissue Cytosols and Assay of Their Enzymatic Activities. Cytosols were prepared from rodent tissues as described (23). QR activities were determined on tissue cytosols by measuring the rate of the NADPH-dependent menadion-mediated reduction of 3-(4,5-diethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide at 610 nm, as described by Prochaska and Santamaria (19), with the following modifications. The assays were conducted at 25°C in 3.0-ml systems containing: 25 mM Tris-Cl (pH 7.4), 50 µM menadione in 10 µl of acetonitrile, 0.75 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 30 µM NADP, 5 µM flavin-adenine dinucleotide, 1 mM glucose 6-phosphate, 5 units of yeast glucose-6-phosphate dehydrogenase, 0.07% bovine serum albumin, and 0.01% Tween 20. GST activities were measured with CDNB and DCNB (24).

Protein concentrations were determined according to the method of Bradford (25) with bovine serum albumin as standard. All enzyme-specific activities are expressed as nmol of product formed per min per mg of protein.

Statistical Treatment. The mean enzyme specific activities for cytosols obtained from 4-6 animal tissues were calculated and the standard errors of the means were computed. The ratios of the mean enzyme specific activities of tissue cytosols from animals treated with inducers to those receiving control diets were then calculated (see Figs. 1 and 2, and Table 1). The standard errors of these ratios were obtained as follows. If the mean specific activity values for groups A and B are Xₐ (±SEMₐ) and Xₗ (±SEMₗ), respectively, the SEM of the ratio Xₐ/Xₗ is:

\[
\text{SEM}_{X_a/X_b} = \sqrt{\left[\frac{\text{SEM}_a}{X_a}\right]^2 + \left[\frac{\text{SEM}_b}{X_b}\right]^2}
\]

RESULTS

Quinone Reductase Induction in Murine Hepatoma Cells. The murine hepatoma Hepa 1c1c7 cell line responds to nearly all known chemoprotectors by severalfold elevation of the specific activities of QR (9, 11, 12). The concentration-dependent inductions of QR in these cells by fumaric and maleic acids and their methyl and ethyl esters are shown in Fig. 1. The esters are much more potent inducers than the acids, as would be expected, because they are more efficient Michael reaction
enzymes and small intestine were increased 3.7- to 9.0-fold.

Significant elevations of QR occurred only at concentrations of the free acids exceeding 50 μM. Dimethyl succinate was inactive as an inducer, again as expected, since it is not a Michael reaction acceptor. The diethyl esters were somewhat less potent than the dimethyl esters. The CD values were 20 and 9.5 μM for diethyl fumarate and diethyl maleate, respectively.

As noted previously (22), the basal QR specific activities of the cytosols of the BPc1 mutant were much lower (26%) and of the c1 mutant much higher (402%) than those of the wild-type Hepa 1c1c7 line. Despite the large differences in basal QR activities, however, the potencies of induction of QR of the dimethyl esters of fumarate and maleate were almost the same in these mutants as in the wild-type cell line (data not shown). In contrast, β-naphthoflavone was not an inducer in the mutants, whereas it is a very potent inducer (CD = 35 nm) in the wild-type Hepa 1c1c7 cells. These findings establish that DIMEFU is a monofunctional inducer (3, 10) that does not depend on a functional Ah receptor or on cytochrome P450 (aryl hydrocarbon hydroxylase) activity for induction.

Enzyme Inductions in Mouse Organs. Addition of 1.0 or 2.0% fumaric acid to the diets of 6-week-old female CD-1 mice for 14 days resulted in normal weight gain and was well tolerated. Survey of tissue cytosols disclosed only minor, although in some tissues, significant elevations of the specific activities of QR and GSTs (data not shown). Since fumaric acid is a relatively poor inducer compared to its esters (9), we next examined the effects of 0.2 and 0.5% DIMEFU under similar conditions. The mice weighed 25.9 ± 1.2 g at the beginning of DIMEFU feeding. The weight gain of the control group was 12% during the 14-day feeding period. The animals receiving DIMEFU gained 10.0 and 1.3% of their initial weight for the groups receiving 0.2 and 0.5% DIMEFU, respectively, and 10.0% for those that received 0.75% BHA.

As previously described in CD-1 mice (27), the basal QR specific activities were 10- to 60-fold higher in nearly all peripheral tissues (except spleen) than in the liver. In contrast, the basal GST specific activities were quite similar in all the tissues examined except for the very low levels in the spleen (28) (see Fig. 2 caption). DIMEFU feeding raised the specific activities of QR and GST in a dose-dependent manner in nearly all organs (Fig. 2). At the higher dose, the ratios of the specific activities of these enzymes (treated/controls) were elevated at least 2.1-fold and as much as 9.0-fold (Fig. 2) in several organs examined, except for the spleen, where the GST activities with DCNB were not measurable, and the colonic mucosa, where this activity was not significantly increased. The greatest elevations of QR in response to DIMEFU were observed in the forestomach and small intestine (4-fold in each tissue), whereas the GST activities in liver, forestomach, glandular stomach, and small intestine were increased 3.7- to 9.0-fold.

In contrast to the effects of feeding 0.75% BHA to female CD-1 mice, which resulted in significant inductions of QR and GST primarily in liver and small intestine (27–29), DIMEFU raised these activities in many tissues and might therefore be expected to exert more widespread protection.

Enzyme Inductions in Rat Organs. Female Sprague-Dawley rats were fed a diet supplemented with 0.2, 0.4, or 0.5% DIMEFU for 14 days beginning at 25 days of age. Control groups received this diet without supplement, and a positive control group received 0.4% ethoxyquin, which is a known inducer of GST in rodent tissues (30). The gains in body weights of the rats at the end of the 14-day feeding period (from an initial weight of 63.7 ± 4.4 g to a final weight of 135.0 ± 5.6 g in the control group) were unaffected by the feeding of 0.2% DIMEFU, but were reduced to 93 and 87% of control values on the 0.4 and 0.5% DIMEFU diets, respectively. Liver weights were similar in rats receiving 0.2% DIMEFU to those of controls, but were lowered by the feeding of 0.4 and 0.5% DIMEFU to 96.3 and 86.9% of control values, respectively. In contrast, whereas 0.4% ethoxyquin treatment decreased final body weights to 95.3% of controls, liver weights were raised to 113% of controls.

The enzyme induction patterns evoked by ethoxyquin and DIMEFU were quite different among the various organs (compare Fig. 3 and Table 1). Thus, the most dramatic effects of ethoxyquin were in the liver (3- to 4-fold inductions of the three
enzymes), whereas the highest doses of DIMEFU produced only a 2.1-fold induction of QR and a 1.39 (CDNB)- to 1.53 (DCNB)-fold increase in GST activities in this organ. In contrast, in the forestomach, 0.4% ethoxyquin raised the enzyme activities 1.19- to 1.75-fold, whereas DIMEFU increased these specific activities 6.0- to 11.3-fold. In all of the organs studied, QR was induced by DIMEFU in a dose-dependent manner. Similarly, GSTs measured with CDNB and with DCNB were raised by DIMEFU, except in the colon where GST activity with CDNB was barely elevated, and GST activity with DCNB was not measurable. Since there are multiple GSTs in rat tissues, and the precise composition of isoenzymes in each tissue following induction by DIMEFU is unknown, it is clear that the response to DIMEFU is complex, and that undoubtedly various types of GSTs are induced differentially, as are the induction patterns evoked by BHA in the liver (31, 32). Nevertheless, the inductions of the Phase 2 enzymes evoked by DIMEFU were much less organ specific than those produced by the antioxidant ethoxyquin, which raised enzymes principally in the liver and small intestinal mucosa (Table 1).

**DISCUSSION**

Well-tolerated levels (0.2–0.5%) of DIMEFU in the diet of female CD-1 mice and female Sprague-Dawley rats elevated the specific activities of chemoprotective enzymes (QR and GSTs) in the liver and several peripheral tissues. Such enzyme induction patterns are characteristic consequences of the administration of many types of compounds (e.g., phenolic antioxidants, isothiocyanates, 1,2-dithiole-3-thiones, thiocarbamates, coumarins) that protect animals against the carcinogenic and other toxic effects of a wide variety of chemical agents (2). Therefore, it seems likely that DIMEFU will be found to be a chemoprotective agent, especially since the chemoprotective function against carcinogenesis has already been demonstrated for fumaric acid (17, 18, 33, 34).

The following considerations make the possibility of developing DIMEFU as a chemoprotector especially attractive: (a) DIMEFU is a monofunctional inducer and it is therefore unlikely to induce Ah receptor-dependent cytochromes P-450 that activate many carcinogens to their ultimate reactive forms, (b) Fumaric acid (17, 18, 33, 34). The function against carcinogenesis has already been demonstrated for fumaric acid (17, 18, 33, 34).

**Table 1** Induction of QR and GST activities by 0.4% dietary ethoxyquin in cytosols of female Sprague-Dawley rat organs

<table>
<thead>
<tr>
<th>Organ</th>
<th>Ratio of specific activities (treated/control)</th>
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<tbody>
<tr>
<td>Liver</td>
<td>CDNB</td>
</tr>
<tr>
<td>Forestomach</td>
<td>CDNB</td>
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<tr>
<td>Glandular stomach</td>
<td>CDNB</td>
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<tr>
<td>Small intestine</td>
<td>CDNB</td>
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<tr>
<td>Colon</td>
<td>CDNB</td>
</tr>
<tr>
<td>Lung</td>
<td>CDNB</td>
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<tr>
<td>Kidney</td>
<td>CDNB</td>
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* ND, not detectable.
studies of the reputed medicinal properties of shepherd’s purse (Capsella bursa-pastoris), a cruciferous weed that has been widely used as a traditional herbal remedy (37-41). The active principle of shepherd’s purse responsible for these activities was isolated and identified as fumaric acid (42). Fumaric acid was then shown to inhibit the growth of solid Ehrlich tumor in mice (41), to inhibit gastric ulceration in rats (42), and to reduce the toxicity of mitomycin C for mice (34) and ameliorate its toxicity for cultured cells (34, 43). The finding that extracts of shepherd’s purse protect rats against the hepatocarcinogenesis of azo dyes in rats (40) and that fumaric acid is a chemoprotector against carcinogenesis in rodents (17, 18) argue for the chemoprotective potential of fumarate esters. A clear-cut causal relation between the protective effects of fumaric acid and the potent induction of Phase 2 enzymes by fumarate esters has not been established. Since, however, chemical protection against the toxic and neoplastic effects of chemical carcinogens is often associated with induction of Phase 2 enzymes, this relation mandates further analysis.

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