Dibenzoylmethane Activates Nrf2-Dependent Detoxification Pathway and Inhibits Benzo(a)pyrene Induced DNA Adducts in Lungs

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Abstract: Cigarette smoke derived carcinogens have been identified as the main agents implicated in lung carcinogenesis. Epidemiological as well as animal studies have indicated that certain phytochemicals can block the carcinogenic process by enhancing the detoxification of environmental and or dietary carcinogens. Dibenzoylmethane (DBM), a minor constituent of licorice, is a beta-ketone analog of curcumin, a promising chemopreventive agent for colon, breast and skin cancer. The present study was designed to examine the chemopreventive efficacy of DBM in lungs, its global molecular targets and the mechanism of its action. Feeding DBM to A/J mice significantly inhibited benzo[a]pyrene induced DNA adducts in lungs. Further analysis of its global molecular targets in lungs by oligonucleotide microarray revealed expression of several cytoprotective genes including phase II enzymes that are regulated by Nrf2, a basic leucine zipper transcription factor. To decipher if DBM mediates its function *via* Nrf2 activation, Nrf2 dependent reporter assays were performed. DBM elicited a dose-dependent increase in antioxidant response element (ARE)-driven luciferase reporter activity which correlated with an increase in mRNA expression of NQO1, GSTA2, and GCLC in mouse hepatoma cells, which are well established targets of Nrf2. Conversely, DBM stimulated ARE reporter activity was attenuated by a dominant-negative mutant of Nrf2. Electrophoretic mobility shift assay confirmed that DBM greatly increased the DNA binding activity of Nrf2. In conclusion, DBM mediates the induction of phase II enzymes by Nrf2 activation and inhibits benzo[a]pyrene induced DNA adducts by enhancing its detoxification in lungs.

Key Words: Dibenzoylmethane, Nrf2, Benzo(a)pyrene, A/J mice, lung, Phase II enzymes, DNA adducts.

INTRODUCTION

Despite several decades of cancer research, the overall mortality rates due to various forms of cancers remain high. On the other hand, over 250 population based-studies indicate that consumption of fruits and vegetables can greatly reduce the incidence of several types of cancers including pulmonary cancer [1]. These reports emphasize the concept of cancer prevention referred as 'chemoprevention' by exploiting phytochemicals (both nutritive and non-nutritive) against cancer. Mechanistic understanding of these phytochemicals both at the molecular and cellular level, is a prerequisite not only for their development as chemopreventive agents but also for targeting against the precise stage of carcinogenesis (e.g., initiation, promotion and progression) [2].

Xenobiotic detoxification enzymes play a major role in combating toxicity, mutagenicity, carcinogenicity as well as oxidative stress associated with environmental or dietary-derived carcinogens by modulating their metabolism. Phase II enzymes such as NAD(P)H:quinone reductase (NQO1), glutathione-*S*-transferase (GST), and UDP-glucuronosyltransferases (UGT) are members of a family of enzymes which are involved in the detoxification of carcinogens. Upregulation of these enzymes greatly protects against various forms of chemical, dietary and cigarette smoke-induced carcinogenesis [3,4] . Conversely, low expression of these enzymes, for example in the case of GSTM2 null genotype, has been associated with a higher risk towards PAH-induced lung cancer. Using transgenic mice, it has been shown that phase II detoxification enzymes such as GST can determine the susceptibility to chemical carcinogenesis [5]. Induction of phase II enzymes by chemopreventive agents is mainly mediated by Nrf2, a bZIP redox sensitive transcription factor [2,6,7]. In response to electrophiles and reactive oxygen species, Nrf2 dissociates from its cytoplasmic repressor, Keap1, translocates to the nucleus, binds to a *cis*-element called the 'antioxidant response element' (ARE) in conjugation with small Maf proteins and plays a central role in the constitutive and inducible expression of phase II enzymes and antioxidants enzymes [6,8]. We and others have reported enhanced susceptibility of Nrf2-deficient mice to butylated-hydroxytoluene-induced toxicity [9], cigarette smoke-induced emphysema [8], allergen-induced asthma [10], LPS-induced acute lung injury [11], bleomycin-induced fibrosis [12], benzo[a]pyrene-induced mutations [13], and diesel exhaust-induced DNA adducts in lungs [14].

Certain phytochemicals have been demonstrated to activate Nrf2 and upregulate innate stress response genes including phase II enzymes and antioxidants that confer protection against carcinogen-induced pro-mutagenic lesions, such as DNA adducts without causing cellular toxicity. Several recent studies have employed this concept for cancer prevention and have found encouraging results [4]. Oltipraz (5-[2-

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pyrazinyl]-4-methyl-1,2-3-thione), an Nrf2 activator, significantly reduced benzo[a]pyrene (B[a]P)-induced forestomach tumors in mice [7]. Clinical trials with oltipraz also showed great potency in altering aflatoxin metabolism leading to increased excretion in urine [15]. Sulforaphane, an inducer of phase II enzymes *via* Nrf2 [2], isolated from broccoli, significantly decreased B[a]P-induced forestomach tumors in mice suggesting the effectiveness of increasing carcinogen detoxification to protect against cancer [16,17]. More recently, synthetic triterpenoids, such as CDDO-Im, was demonstrated to inhibit aflatoxin-induced carcinogenesis by phase II induction through Nrf2 [18]. Anethole dithiolethione, another inducer of phase II enzymes [19], has been recently shown to be effective in a randomized phase IIb trial of former and current smokers with bronchial dysplasia [20]. Such a strategy reiterates that the induction of innate stress response genes confers resistance against carcinogen-induced toxicity and oxidative stress.

The lung is the main target organ for all the inhaled environmental toxicants. Upregulation of lung cytoprotective defenses including phase II enzymes and antioxidants can help counteract the alterations in cellular homeostasis due to xenobiotic toxicities. However, not much success has been achieved in identifying agents that can upregulate phase II enzymes in the lungs. Poor bioavailability of chemopreventive agents in the lung has been one of the factors attributed to this failure. Blocking agents, such as phenethyl isothiocyanate, benzyl isothiocyanate and green tea polyphenols provide significant protection against polyaromatic hydrocarbons, such as 4-(N-methyl-N-nitrosamino)-1- (3-pyridyl)-1-butanone (B[a]P)-induced lung cancer but failed to show a protective effect against tobacco smoke-induced lung cancer [21]. Therefore, the discovery of novel chemopreventive agents with the potency to upregulate cellular cytoprotective systems in lungs is needed.

Curcumin (diferuloylmethane), a phenolic antioxidant, has been employed as a chemopreventive agent for skin and colon cancer [22,23]. However, its chemoprotective role in the lungs is poor [24]. DBM is a curcumin analog and a minor phenolic constituent of licorice. DBM has been shown to be more potent than curcumin in inducing quinone reductase activity in mouse hepatoma cells [25]. Both compounds have a β-diketone group and conjugated double bonds; however, DBM has a smaller molecular size and lacks hydroxyl groups (Fig. **1**). It has been demonstrated that DBM inhibits the mutagenicity of 2-naphtholhydroxamic acid, B[a]P and aflatoxin B1 in *Salmonella typhimurium* [26]. In addition, DBM protects against 7,12- dimethylbenz[a]anthracene (DMBA)-induced breast cancer in a mouse model, and showed strong anti-inflammatory and anti-tumor promoting activities in mouse skin [27]. Unlike curcumin, the molecular mechanism of its action and its effect in the lungs need further evaluation.

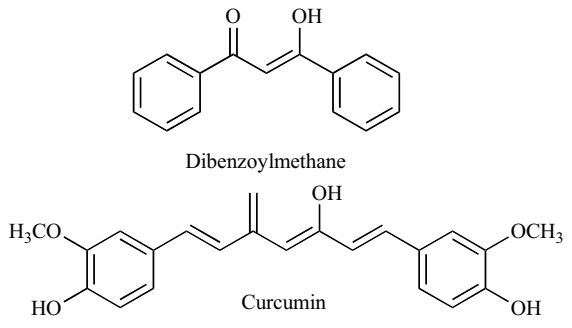


Fig. (1). Structure of dibenzoylmethane and curcumin.

The present study was designed to elucidate the chemopreventive efficacy of DBM in the lung, its global molecular targets and the underlying molecular mechanism of action. The results demonstrate the potency of DBM to inhibit B[a]P-induced DNA adducts in mice lungs. DBM mediates its effect by up-regulating several cytoprotective genes including phase II enzymes mainly *via* Nrf2 activation.

RESULTS

Inhibition of B[a]P-Induced DNA Adducts in Lungs by DBM

To determine the potency of DBM to protect against carcinogen-induced DNA damage in lungs, mice were orally fed DBM for 3 days followed by a single dose of B[a]P. DBM greatly reduced B[a]P-induced DNA adducts in lungs measured after 48 h of B[a]P treatment. The percent decrease in the levels of B[a]P-induced DNA adducts, as measured by ^{32}P post-labeling in the DBM fed group, was 60 % as compared to the group treated with B[a]P alone (Fig. (**2a**) & (**b**)). There were no significant changes in body weight due to DBM treatment (data not shown).

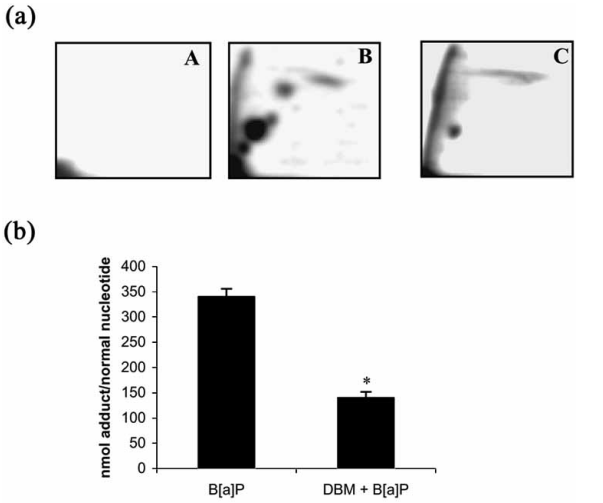


Fig. (2). ^{32}P-postlabelling analysis of B[a]P-DNA adducts in A/J mice lungs.

a) Representative autoradiograms of PEI-TLC maps depicting B[a]P-DNA adducts in the A/J mice lungs treated with (A) vehicle; (B) B[a]P; (C) DBM and B[a]P. **b**) Levels of B[a]P induced DNA adducts in lungs of mice fed with DBM at a dose of 500 mg/kg body weight for 5 days prior to B[a]P (1 mg/kg body weight) administration. Values represent mean ± SE (n=3). * Significantly different from control ($P \leq 0.05$).

Global Molecular Targets of DBM in Lungs

In order to decipher the global molecular targets of DBM in the lung, a global gene expression profile from mice fed with or without DBM was generated using an oligonucleo-

tide microarray approach. After DBM treatment for 3 days, 208 genes were up-regulated and 160 genes were down-regulated. Genes were broadly classified based on their biological function. DBM induced several xenobiotic detoxification enzymes including GST and UGT, which are involved in the detoxification of B[a]P. Genes associated with anti-oxidant defenses, such as glutamate-cysteine ligase (catalytic subunit) (GCLC), glutathione peroxidase and thioredoxin reductase were also upregulated. Apart from these cytoprotective genes, heat shock proteins, transcription factors, and various other genes were upregulated (Table **1**).

Validation of Microarray Data by Northern Blot

Oligonucleotide microarray data was validated by measuring the transcriptional changes of selected genes specifically NQO1, GST a2 and GCLC through Northern hybridization. The mRNA levels of NQO1, GST (Ya) and GCLC, in the lungs of DBM fed mice were higher by 3.2 ± 0.15, 3.4 ± 0.31 and 3.6 ± 0.42 fold respectively than the control group (Fig. (**3**)) which is consistent with the microarray data.

NQO1 and GST Enzyme Activity

Along with transcript levels, enzymatic activity of NQO1, and GST was also evaluated in the lungs following DBM treatment. At 24 h after the last dose of DBM, there was a significant increase in GST (45%) and NQO1 (51%) activity in the lungs compared to the vehicle fed control group (Fig. (**4**)).

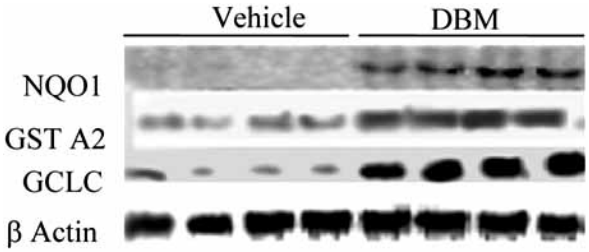


Fig. (3). Transcript levels of NQO1, GST A2 and GCLC in lungs of mice treated with DBM. Mice were fed with DBM at a dose of 500 mg/kg body weight for 5 days. After 24 h of the last dose, lungs were excised, total RNA was extracted and mRNA level of NQO1, GST A2 and GCLC were measured using Northern hybridization. Levels of lung mRNA for each gene were normalized to β- actin mRNA levels.

Activation of Nrf2 by DBM

Phase II enzymes have been shown to be primarily regulated by Nrf2. Nrf2, in response to stimuli, translocates to the nucleus, binds to the ARE along with other partners and transactivates gene expression. DBM induced expression of several phase II genes in the lung. In order to understand if DBM mediates its action through Nrf2 activation, Nrf2-mediated reporter assays were performed using mouse Hepa cells stably transfected with an ARE-controlled firefly luciferase reporter construct, pARE-luc. Hepa cells exposed to varying concentrations (5, 10 & 50 μM) of DBM revealed a

Table 1. DBM Induced Selected Cytoprotective Genes in the Mice Lungs Obtained by Oligonucleotide Microarray

Gene Title	FC ± SE*	Gene Title	FC ± SE
Antioxidants / Detoxification enzymes		Chaperones/proteosome	
Aldehyde oxidase 1	1.66 ± 0.41	Heat shock protein, 70 kDa 1	4.96 ± 0.73
Carboxylesterase 1	6.06 ± 0.66	Heat shock protein, 70 kDa 3	4.86 ± 0.71
Cytochrome P450, 2a5	2.36 ± 0.42	Heat shock protein, 86 kDa 1	1.41 ± 0.35
Flavin containing monooxygenase 2	1.53 ± 0.39	Heat shock protein 105 kDa	2.69 ± 0.42
Flavin containing monooxygenase 3	1.40 ± 0.38	Heat shock protein HSP27 internal deletion variant b	1.61 ± 0.37
Glutamate-cysteine ligase, catalytic subunit	1.40 ± 0.41	Heat shock protein, 25 kDa	1.53 ± 0.36
Glutathione peroxidase 2	1.87 ± 0.45	DnaJ (Hsp40) homolog, subfamily B, member 1	1.87 ± 0.42
Glutathione S-transferase, alpha 2	2.36 ± 0.53	Ubiquitin specific protease 2	1.54 ± 0.40
Glutathione S-transferase, alpha 3	1.96 ± 0.40	DnaJ (Hsp40) homolog, subfamily A, member 1	1.56 ± 0.39
Glutathione S-transferase, mu 1	1.72 ± 0.39	Crystallin, alpha C	1.47 ± 0.37
Glutathione S-transferase, mu 3	4.69 ± 0.65		
NAD(P)H dehydrogenase, quinone 1	2.41 ± 0.58		
Thioredoxin reductase 1	1.67 ± 0.41		
UDP-glucuronosyltransferase 1 family, member 1	1.44 ± 0.39		
Phenol UDP-glucuronosyltransferase,	1.60 ± 0.36		

* FC, Fold change; SE, Standard error.

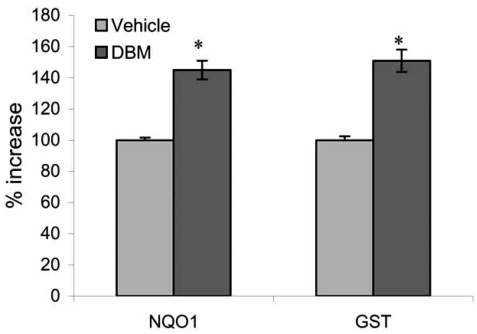
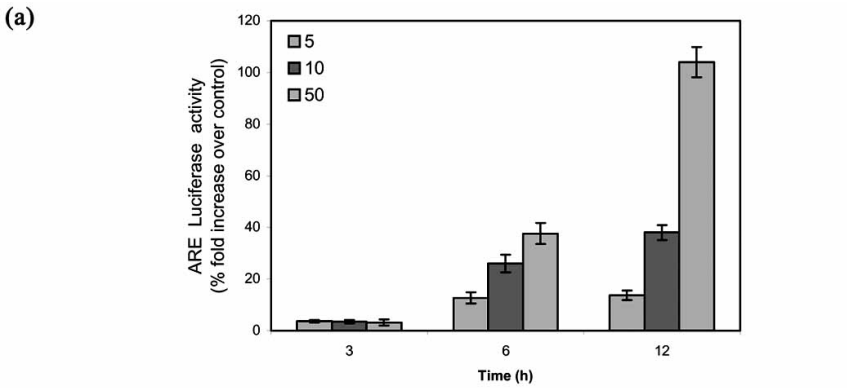


Fig. (4). NQO1 and total GST enzyme activity in lungs of A/J mice fed on DBM for 5 days (n=4). Values are present as mean ± SE. * Significantly different from control ($P \leq 0.05$).

dose-dependent increase in luciferase activity as early as 6 h post-treatment (Fig. (**5a**)). The ARE reporter activity increased by 40-fold and 100-fold after 6 h and 12 h of exposure to 50 μM DBM, respectively. Co-transfection with a dominant-negative Nrf2 construct greatly diminished the ARE reporter activity in DBM treated Hepa cells (Fig. (**5b**)) confirming the role of Nrf2 in activating the ARE. Co-transfection either with pRJB- c-Jun and or a dominant negative c-Jun construct showed no significant effect on luciferase activity when compared to its parent vector. To further confirm that DBM activates Nrf2, nuclear extracts prepared from Hepa cells treated with 50 μM DBM for 2 h were analyzed by EMSA using the consensus sequence of human NQO1 ARE to assess the DNA binding activity of Nrf2 and c-Jun respectively. A significant increase in the DNA binding activity of Nrf2 was observed with nuclear extracts from DBM treated cells when compared to the vehicle treated controls, suggesting the activation of Nrf2 by DBM in the Hepa cells (Fig. (**6**)).



DBM	-	+	+	+	+	+	+	+
Renilla	-	-	+	+	+	+	+	+
pEF	-	-	+	-	-	-	-	-
pRJB10B	-	-	-	+	-	-	-	-
pEF-Nrf2	-	-	-	-	+	-	-	-
pEF-Nrf2M	-	-	-	-	-	+	-	-
pRJB-cJun	-	-	-	-	-	-	+	-
pEF-cJunM	-	-	-	-	-	-	-	+

Fig. (5). DBM enhances Nrf2 mediated ARE luciferase reporter assay. (a) Increase in luciferase activity in Hepa cells treated with DBM. Hepa cells, stably transfected with pARE-luc, were treated with DBM at varying concentration (5, 10 or 50 μM). At 6, 12, & 24 h post-treatment, luciferase activity was measured using the luciferase reporter assay. The data represented mean ± SE of percent increase over control is from 3 independent experiments; (b) A dominant-negative mutant of Nrf2 inhibits DBM induced luciferase activity in Hepa cells. The cells were transfected either with Nrf2 (pEF-Nrf2), c-jun (pRJB-c-Jun), dominant-negative Nrf2 (pEF-Nrf2M), dominant negative c-Jun (pEF-c-JunM) and or its parent vector (pEF or pRJB10B) as described in the methods. 24 hours post-transfection, the cells were treated with 50μM DBM and the luciferase activity was measured using the dual luciferase reporter assay after 12 hours post-treatment. The data were expressed as firefly luciferase activity normalized against the renilla luciferase activity. The data represented are mean ± SE of three independent experiments (*a*- significant compared to parent vector*; b*- significant compared to pEF-Nrf2).

Fig. (6). Increase in DNA binding activity of Nrf2 in Hepa cells after DBM treatment as determined by electrophoretic mobility shift assay. Hepa cells were incubated with either vehicle or DBM (50μM) for 2h at 37 ^{0}C. Nuclear extracts were prepared, and the binding to the consensus sequences of human NQO1 ARE was determined by EMSA.

In corroboration with the reporter assay, Hepa cells exposed to DBM showed a dose and time dependent increase in the level of NQO1, GST a2 and GCLC mRNA expression (Fig. (**7**)). Treatment of Hepa cells with DBM also demonstrated a dose and time dependent increase in the enzyme activity of NQO1. The increase in enzyme specific activity at 10 and 50 μM DBM after 12 h was 2.5-fold, which further increased to 5 and 7- fold after 24 h respectively (Fig. (**8**)).

DISCUSSION

Curcumin has been well established in experimental studies to have anti-inflammatory properties, prevent tumorigenesis and mutagenesis, block carcinogen DNA adducts formation, and inhibit angiogenesis [28-31]. Furthermore, it has been reported that curcumin is an inducer of phase II enzymes in the liver [32]. However, its potency in the lung is poor. Hence, great attention has been focused towards natural and synthetic analogs. These analogs show greater potency in terms of phase II enzyme induction in cell culture, although their functional potency in animal models have not been elucidated [25]. DBM is a chemical analog of curcumin, which has shown promising results against breast cancer in a rodent carcinogenesis model. In contrast to curcumin, DBM showed weak *in vitro* antioxidant potency, however it showed strong anti-inflammatory and anti-tumor promoting activities in mouse skin [27,33,34]. In addition dietary DBM inhibited DMBA-induced mammary tumorigenesis in sencar mice and rats whereas curcumin had little or no inhibitory effect [27,35]. Currently, the role of DBM against lung cancer has not been investigated. Derivatives of DBM occur in several plant families, mainly bearing isoprenoid- or furano-substitutions on the aromatic rings, or more rarely an allyl-group bonded to the central carbon of an aliphatic chain [36]. Existing exclusively as keto-enolic tautomers, such molecules have been described as `ß-hydroxychalcones'. These molecules accumulate upon pathogen attack and are potent antimutagens [26] and are powerful sunscreens in the UVA region.

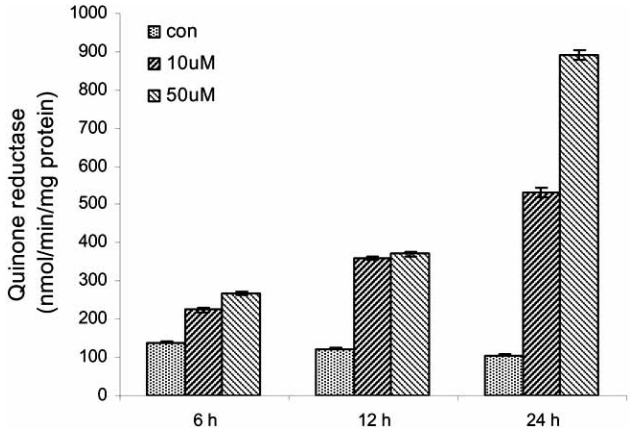


Fig. (8). Dose- and time-dependent profile of NQO1 enzyme activity in Hepa cells treated with DBM. Values represent mean ± SE. * Significantly different from control ($P \leq 0.05$).

In the present study, we have demonstrated the chemopreventive efficacy of DBM by measuring B[a]P-induced DNA adducts, an intermediate biomarker of carcinogenesis in lungs of A/J mice, which is a strain that is sensitive to B[a]P-induced lung carcinogenesis [37] . When DBM was fed orally for 3 days to mice prior to B[a]P administration, DBM significantly inhibited B[a]P-induced DNA adducts in lungs while curcumin at similar doses showed a moderate inhibition of B[a]P-induced DNA adducts in lungs (supplemental data Fig. (**1**)). The observed response is similar to DMBA-DNA adduct formation in rat mammary tissue [33].

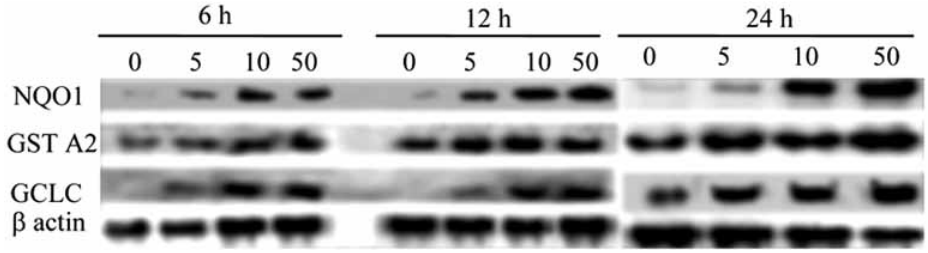


Fig. (7). Dose- and time-dependent increase in mRNA expression of NQO1, GST (Ya) and GCS (h) in Hepa cells treated with DBM. Cells were treated with 5, 10, or 50μM of DBM and total RNA was collected after 6, 12, and 24 h treatment. The mRNA levels of NQO1, GST (Ya) and GCS (h) were measured using Northern hybridization. RNA levels for each gene were normalized to β- actin mRNA levels.

The ability of DBM to inhibit B[a]P-induced DNA adducts have also been established in human mammary epithelial cells [38]. Curcumin has been demonstrated to have no effect on B[a]P and NNK-induced lung tumors although it shows good protection against B[a]P-induced forestomach tumorigenesis. The weak chemopreventive potency of curcumin in lung tissue is probably due to the poor absorption and bioavailability [39]. Unlike curcumin, induction of phase II genes by DBM highlights its bio-availability in the lungs (Fig. (**3**) and supplemental data Fig. (**2**)).

The gene expression profile induced by DBM in mice lungs reveals its pharmacological response. Microarray data revealed upregulation of several cytoprotective genes including genes associated with xenobiotic detoxification and antioxidants in lungs in response to DBM feeding. Several isoforms of GST (GSTa2, GSTa3, GSTm1, GSTm3) were upregulated. GSTs constitute a family of enzymes that play important role in cellular defense against carcinogenic effects of a wide range of polyaromatic hydrocarbons. The tumorigenic activity of B[a]P is mainly attributed to its metabolite benzo[a]pyrene-7, 8-diol-9, 10-epoxide (BPDE). GSH conjugation to BPDE, catalyzed by GST, is the main pathway for B[a]P detoxification. The protective role of GST alpha and mu isoforms, towards B[a]P-induced DNA adducts have been well recognized [40,41]. NQO1 was also upregulated in mice lungs fed DBM. NQO1 is involved in the detoxification of quinones and its derivatives, which are contributors of oxidative stress. NQO1 has been shown to suppress benzopyrene quinone DNA adducts [42]. UGT is another important enzyme upregulated in lungs that increases detoxification of PAH including B[a]P by glucuronidation. The observed decrease in B[a]P-induced DNA adducts in lungs of mice fed with DBM could be the result of synergistic effect of all these detoxification enzymes. Other detoxification enzymes upregulated by DBM in lungs are carboxylesterase, aldehyde oxidase 1, flavin containing monooxygenase 2, flavin containing monooxygenase 3 and cytochrome P450, 2a5. In addition to xenobiotic metabolic enzymes, DBM upregulated antioxidants genes, such as glutathione peroxidase, thioredoxin reductase and GCLC. Glutathione peroxidase reduces hydrogen peroxides and lipid hydroperoxides by consuming GSH while GCLC is one of the rate limiting enzymes in GSH biosynthesis. Thioredoxin reductase in association with thioredoxin plays an important role in cellular antioxidant defenses and redox regulation [43].

DBM upregulated several heat shock proteins (HSPs) namely HSP 70, HSP 86, HSP 25, HSP 105 and HSP 40. HSP, also called stress proteins, protect the cell from various exogenous and endogenous stresses. Also the molecular chaperone activity of HSPs play a major role in regulating the conformation and activity of a variety of cellular proteins [44,45].

Our gene expression study, as well as studies from other laboratories, clearly indicates the potency of DBM to induce phase II enzymes. The transcriptional activation of phase II enzymes has been demonstrated to be regulated by a *cis*-acting enhancer, ARE. By employing gene disrupted mice models, the transcription factor, Nrf2 has been shown to regulate ARE-mediated expression of phase II enzymes in response to electrophiles and reactive oxygen species [46,47]. Increased sensitivity of Nrf2-null mice to B[a]P-induced DNA adducts in the lung, liver and stomach has established the pivotal role of Nrf2 in protection against chemical carcinogenesis [7,14,48] and also underscores the importance of Nrf2 activators in chemoprevention studies [7]. Further, several studies have demonstrate that Nrf2 plays a central role in multi-organ (lung, liver, kidney, stomach, small intestine, central nervous system) protection from a wide array of oxidants and inflammatory agents [11,49]. In order to elucidate if DBM mediates induction of phase II enzymes *via* Nrf2 activation, mechanistic studies were conducted in mouse Hepa cells that were stably transfected with an ARE-dependent luciferase construct. In response to DBM, there was a time- and dose-dependent induction of NQO1, GST Ya and GCLC in the Hepa cells (Fig. (**3**) & (**5**)). DBM caused an increase in ARE luciferase reporter activity (Fig. (**5a**)) which correlated with an increase in proteins from nuclear extract binding to ARE sequence as evidenced from the EMSA results (Fig. (**6**)). The importance of Nrf2 in this response was underscored by the inhibitory effect of a dominant-negative Nrf2 on DBM-mediated ARE activation in Hepa cells (Fig. (**5b**)). Although, all the components binding to ARE are far from clear, transcriptional regulators such as Nrf2-maf heterodimer, p160 family coactivator, ARE-binding protein 1, CBP/p300 have been shown to be few members that bind to ARE [50]. Nrf2 is held in cytoplasm by its repressor protein Keap1. Recent reports suggest that dissociation of Nrf2 from keap1 can occur by two mechanisms, 1) phosphorylaton of Nrf2 by upstream signaling protein kinases including protein kinase C, mitogen-activated protein kinase, phosphatidylinositol 3-kinase, and PKR-like endoplasmic reticulum kinase [6]; 2) direct modification of cysteine residues in Keap1 by the activators that causes in conformation change rendering dissociation of Nrf2 [6] . Our study demonstrates that DBM increases the binding of Nrf2 with the ARE, however, the underlying mechanisms of Nrf2 dissociation with Keap1 needs further investigation.

In conclusion, this study demonstrates the efficacy of DBM to upregulate several cytoprotective genes including phase II enzymes *via* Nrf2 activation. The results suggest that DBM is a promising chemopreventive agent that can confer protection against a wide range of lung carcinogens.

MATERIALS AND METHODS

Reagents

Dibenzoylmethane, curcumin, NADPH, glutathione, 1-chloro-2,4-dinitrobenzene, menadione and benzo(a)pyrene were obtained from Sigma Chemical Company (St. Louis, MO). Dulbecco's Modified Eagle's Medium (DMEM), Hanks balanced salt solution (HBSS) was purchased from Invitrogen (Carlsbad, CA). The DC protein assay reagent was from Biorad (Hercules, CA). Anti-Nrf2 antibody (polyclonal, rabbit) was purchased from Santa Cruz Biotechnology, CA. The gel shift 5X binding buffer and luciferase assay kit were purchased from Promega Corporation (Madison, WI). γ-^{32}P ATP and α-^{32}P dCTP were procured from ICN pharmaceutical, CA; Polynucleotide kinase from Roche Molecular Biochemicals, IN. The nucleotide removal kit was from Qiagen,

CA. All oligonucleotides were synthesized at Invitrogen. All other chemicals were of analytical grade.

Animals and Treatment

All experimental protocols conducted in mice were performed in accordance with the standards established by the US Animal Welfare Acts, set forth in NIH guidelines and the Policy and Procedures Manual of Johns Hopkins University Animal Care and Use Committee. Female A/J mice, 7 weeks of age were maintained on AIN 76A diet (Harlan Tekland, Madison, WI) and water *ad libitum.* They were housed at a temperature range of 20-23°C under 12 h light /dark cycles. The mice were divided into 2 groups (n=4): I – Vehicle Control and II- DBM (500 mg/kg body weight). The test compounds were administered by gavage in 0.2 ml corn oil for 3 consecutive days. Body weights were recorded to monitor the health of animals.

^{32}P-Postlabeling

To analyze and quantify the B[a]P induced DNA adducts, mice (n=3) were treated with B[a]P (1 mg/mouse; gavage) 1h after the last dose of the test compound. Animals were sacrificed by cervical dislocation after 48 h of B[a]P treatment. The lungs were removed and rinsed in ice cold PBS and processed for DNA extraction.

DNA was isolated as described earlier with modification [51]. In brief, the tissues were homogenized in 1% sodium dodecyl sulfate with 1mM EDTA and incubated with proteinase K at 37 ^{o}C for 40 min. The homogenates were extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1) and then once with chloroform/isoamyl alcohol (24:1). DNA was precipitated with ethanol and dissolved in 100 mM Tris-HCl buffer pH 8.0 and 1 mM EDTA. The DNA was incubated with RNase A (100 μg/ml) and RNase T1 (2000 U) at 37 ^{o}C for 40 min followed by extraction with chloroform/isoamyl alcohol and precipitated with ethanol. DNA was dried under a stream of nitrogen and stored at –20°C. DNA adducts were analyzed by the ^{32}P -postlabeling method of Reddy and Randerath [52]. Briefly, 10 μg of DNA was digested with micrococcal nuclease (200 mU) and 2 μg of spleen phosphodiesterase at 37°C for 3.5 h. The dephosphorylation of normal nucleotides was carried out at 37 °C for 40 min by adding 3 μg of nuclease P1. The DNA digest was postlabeled with 100 μCi γ- ^{32}P ATP (3000–6000 Ci/mmol) and 4.8 U of polynucleotide kinase at 37°C for 40 h. Unreacted [γ-^{32}P] ATP was degraded by the addition of potato apyrase. The labeled DNA adducts were resolved by multidirectional polyethyleneimine (PEI)-cellulose thin layer chromatography using the following solvents: D1 = 1 M sodium phosphate (pH 6.0); D3 = 4 M lithium formate / 7 M urea (pH 3.5); D4 = isopropanal:4 M ammonium hydroxide (1.1:1); and D5 = 1 M sodium phosphate (pH 6.0). The radioactive areas were located by autoradiography using intensifying screens. The adduct and blank areas were excised from the TLC plate and quantified by Cerenkov counting. Autoradiography was carried out by exposure to Kodak XR-5 films at –70^{o}C for two days. Adduct levels were calculated from the amount of radioactivity on the chromatograms, the amount of DNA digested and the specific activity of the [γ-^{32}P] ATP used for the labeling.

Transcriptional Profiling by Oligonucleotide Microarray

Following 24 h of last dose of DBM, total RNA was extracted from lungs by using TRIzol reagent. The RNA isolated was used for experiments with Mouse Genome 430 version 2 arrays (Affymetrix, Santa Clara, CA) by following the procedure as described earlier [2]. To identify the differentially expressed transcripts, pairwise comparison analyses were carried out with Data Mining Tool 3.0 (Affymetrix). Only those differentially expressed genes that appeared in at least 6 of the 9 comparisons with a fold change greater than 1.4 were selected. In addition, the Mann-Whitney pairwise comparison test was performed to rank the results by concordance as a calculation of significance (P value $\leq$ 0.05) of each identified change in gene expression.

Northern Blotting

Total RNA (10 μg) was separated on 1.2% agarose gels containing 2.2 M formaldehyde, transferred to nylon membranes (Nytran super charge, Schleicher & Schuell, Germany) and ultraviolet-crosslinked. Probes for NQO1, GST a2, GCLC and β-actin were generated by PCR from the cDNA of murine liver as described before [2]. These PCR products were radiolabeled with [α-^{32}P] dCTP using a random primers DNA labeling kit (Invitrogen, CA). Northern hybridization was done using QuickHyb (Stratagene, Carlsbad, CA) as per the manufacturer's protocol. After hybridization, the membranes were washed twice in 0.2% SSC (1X SSC= 0.15 M NaCl/0.015 M sodium citrate) containing 0.1% (w/v) SDS at room temperature for 15 min and finally in 0.1% SSC/0.1% SDS at 60 °C for 45 min. The membranes were exposed to a phosphoimager screen and radioactivity visualized and quantified with a BAS1000 Bioimaging system (Fuji Photo Film, Tokyo, Japan). Levels of RNA were quantified and normalized for RNA loading by stripping and reprobing the blots with a probe for β-actin.

Enzyme Assays

Total GST activity was measured in cytosolic fractions (10,000 x *g*) in the presence of 0.1% bovine serum albumin with 1-chloro-2,4-dinitrobenzene as a substrate [53]; whereas, NQO1 activity was determined by menadione as substrate [54]. Protein concentration was determined by using the Bio-rad DC reagent and bovine serum albumin as the standard.

Cell Culture

Mouse hepatoma (Hepa) cells stably transfected with pARE-luc were cultured in DMEM (pH 7.4) supplemented with 10 % (v/v) FBS, 100 mg/L gentamicin and genetisin. Cells were maintained at 37˚ C with 5 % CO_2. Cultures were passaged at confluency (approximately every three days), trypsinized and counted with a hemocytometer. 1 x 10^6 cells were plated in 6 well dishes and grown for 24 h prior to DBM treatment (dissolved in DMSO). The concentration of DMSO did not exceed 0.1%. The ARE-dependent reporter plasmid, pARE-luc (also referred to as p3XStREluc [55], contains the firefly luciferase gene under the control of three copies of an ARE from the mouse heme oxygenase-1 gene.

ARE Reporter Assay

Hepa cells stably transfected with pARE-luc were grown for 24 hours and treated with varying concentrations (5, 10 &

50 μM) of DBM. The reporter activity was measured with a luciferase assay kit (Promega, Madison, WI) after 3, 6 and 12 h exposure. The luciferase activity between the treatments groups were normalized against protein concentrations measured using Biorad DC reagent. In dominant negative ARE reporter assay, the cells were transfected either with Nrf2 (pEF-Nrf2), c-jun (pRJB-cJun), dominant-negative Nrf2 (pEF-Nrf2 M), dominant negative cJun and or its parent vector (pEF or pRJB10B) [56]. Plasmid pRL-TK (0.5 μg), encoding renilla luciferase, was used as an internal control for normalization of the firefly luciferase activity. A total of 2 μg plasmid DNA/well was used for transfection. The cells were grown for 12 hours and transfected using Panvera TransIT kit. 24 hours post-transfection, the cells were treated with 50 μM DBM. The luciferase activity was measured using the dual luciferase reporter assay at 12 hours post-treatment. The data were expressed as firefly luciferase activity normalized against the renilla luciferase activity.

Electrophoretic Mobility Shift Assay (EMSA)

The oligos were annealed and the nuclear extract was prepared as described before [57]. Human NQO1 ARE sequence was labeled with γ-^{32}P ATP by T4 polynuclotide kinase and purified using the nucleotide removal kit. 10 μg of nuclear extract was incubated with the labeled oligo, analyzed on 5% non-denaturing polyacrylamide gel and developed by autoradiography.

Statistics

The values are represented as mean ± SE and analyzed by student's t-test. Differences were considered significant at $P \leq 0.05$.

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