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Sulforaphane, a cancer chemopreventive agent, induces pathways associated with membrane biosynthesis in response to tissue damage by aflatoxin B_1

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ABSTRACT

Aflatoxin B_1 (AFB_1) is one of the major risk factors for liver cancer globally. A recent study showed that sulforaphane (SF), a potent inducer of phase II enzymes that occurs naturally in widely consumed vegetables, effectively induces hepatic glutathione *S*-transferases (GSTs) and reduces levels of hepatic AFB_1-DNA adducts in AFB_1-exposed Sprague Dawley rats. The present study characterized the effects of SF pretreatment on global gene expression in the livers of similarly treated male rats. Combined treatment with AFB_1 and SF caused reprogramming of a network of genes involved in signal transduction and transcription. Changes in gene regulation were observable 4 h after AFB_1 administration in SF-pretreated animals and may reflect regeneration of cells in the wake of AFB_1-induced hepatotoxicity. At 24 h after AFB_1 administration, significant induction of genes that play roles in cellular lipid metabolism and acetyl-CoA biosynthesis was detected in SF-pretreated AFB_1-dosed rats. Induction of this group of genes may indicate a metabolic shift toward glycolysis and fatty acid synthesis to generate and maintain pools of intermediate molecules required for tissue repair, cell growth and compensatory hepatic cell proliferation. Collectively, gene expression data from this study provide insights into molecular mechanisms underlying the protective effects of SF against AFB_1 hepatotoxicity and hepatocarcinogenicity, in addition to the chemopreventive activity of this compound as a GST inducer.

Introduction

Aflatoxin B_1 (AFB_1) is a known human carcinogen that significantly contributes to the burden of hepatocellular carcinoma (HCC) in many parts of the world, especially in areas with a warm and moist climate such as Asia and sub-Saharan Africa (Kensler et al., 2011). A critical mechanism responsible for the hepatotoxic and carcinogenic potential of AFB_1 is based on the balance of its bioactivation and detoxification (Fig. 1). Several lines of evidence indicate that variation in the extent of glutathione (GSH) conjugation of the ultimate carcinogen, AFB_1-8,9-epoxide, by glutathione *S*-transferases (GSTs) is an important detoxification pathway. Treatment of rats with oltipraz and 3*H*-1,2-dithiole-3-thione (D3T) leads to marked increases in the activity of liver GSTs, which resulted in reductions in both the extent of aflatoxin-DNA adduction and tumorigenesis (Kensler et al., 1987). The inducible A5 subunit of alpha-class GSTs in the rat has been identified as the GST isozyme that is primarily responsible for the enhanced detoxification of the AFB_1-8,9-epoxide by chemopreventive agents (Hayes et al., 1991; Hayes et al., 1998). Modulation of GST activity is only one mechanism by which exogenous agents can influence aflatoxin carcinogenesis. This paper probes additional pathways.

Sulforaphane (SF), a potent isothiocyanate derivative found in broccoli and other cruciferous vegetables (Fahey et al., 1997), has received attention as a chemopreventive agent due to its ability to activate the transcription factor Nrf2 and induce phase II detoxification enzymes, including the GSTs (Fimognari and Hrelia, 2007; Myzak and Dashwood, 2006; Thimmulappa et al., 2002). Recent evidence illustrates protective effects of SF against AFB_1-induced hepatotoxicity in rats attributable to increased GST expression. Treatment of rats with SF resulted in significant induction of hepatic total GST activity and a proportional reduction in the amounts of AFB_1-N^7-guanine (the principal DNA adduct of AFB_1) formed in liver DNA (Fiala et al., 2011). Previous studies, however, have also identified additional chemopreventive mechanisms for SF that are independent of phase II enzyme induction. SF induces apoptosis in both *in vitro* (Fimognari et al., 2004; Karmakar et al., 2006) and *in vivo* models (Singh et al., 2004). SF-mediated cell cycle arrest has been reported in many previous studies, including induction of a dose-dependent growth arrest in prostate cancer cells by inhibiting the

Abbreviations: SF, sulforaphane; AFB_1, aflatoxin B_1; GST, glutathione S-transferase.

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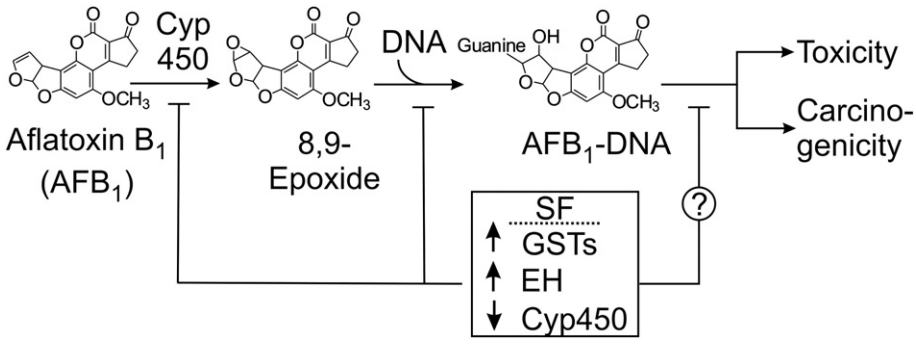

Fig. 1. Pathway of metabolic activation of aflatoxin B_1 and disruption of the pathway by sulforaphane (SF). Aflatoxin is metabolized by cytochrome P450 (Cyp450) oxidases to the 8,9-epoxide, which reacts with DNA to form DNA adducts with guanine residues. The guanine adducts are presumably the functional precursors to carcinogenesis and significant contributors to toxicity. SF induces glutathione transferases (GSTs) and epoxide hydrolases (EH) and downregulates certain Cyp450s. Each of these alterations in enzyme activities would affect the intracellular concentration of the epoxide, hence modulating the levels of DNA adducts.

expression of cyclin D1 and DNA synthesis, along with a G1 cell cycle block (Chiao et al., 2002) and induction of G2/M accumulation and pre-metaphase arrest in bovine aortic endothelial (BAE) cells (Jackson et al., 2007). SF also exerts anti-inflammatory properties by inhibiting pro-inflammatory and pro-carcinogenic signaling factors such as IL-1β (Lin et al., 2008), COX-2 and TNF-α (Heiss et al., 2001). Understanding these phase II-independent pathways provides the rationale for the current project.

Cell regeneration and survival responses signal metabolic reprogramming that supports anabolic pathways required for tissue repair and growth (Ward and Thompson, 2012). The Keap1–Nrf2 complex, which is activated by SF, has been demonstrated to influence intermediary metabolism (Hayes and Dinkova-Kostova, 2014). This study aimed to assess the extent to which anabolic pathways modulated by SF provide protective mechanisms against AFB_1 toxicity *in vivo*. The results revealed prominent reprogramming of gene sets involved in lipid synthesis in SF-pretreated rats, suggesting that SF facilitates regeneration of hepatic cells damaged by AFB_1.

Materials and methods

Chemicals. *R,S*-Sulforaphane (SF) was purchased from LKT Laboratories (St. Paul, MN). Aflatoxin B_1 (AFB_1) was purchased from Sigma Chemical (St. Louis, MO). RNA*later*, RNeasy Mini Kit, and One-Step QuantiTech SYBR Green RT-PCR were obtained from QIAGEN (Valencia, CA). One-Cycle Target Labeling and Control Reagents complete kit (P/N 900493) and GeneChip® Rat Genome 230 2.0 arrays (Rat 230 2.0) were obtained from Affymetrix, Inc. (Santa Clara, CA). R-Phycoerythrinstreptavidin (SAPE) was purchased from Molecular Probes (Eugene, OR) and biotinylated, anti-streptavidin goat antibody was purchased from Vector Laboratories (Burlingame, CA). Reagent grade goat IgG was purchased from Sigma-Aldrich (St. Louis, MO). Nuclease-free water was obtained from Ambion (Austin, TX). DNA primers for real time RT-PCR were ordered from Integrated DNA Technologies (Coralville, IA). Unless otherwise noted, all other chemicals and reagents were of ACS grade or better.

Animals. Male Sprague Dawley rats (21 days old; Charles River, Wilmington, MA) were fed the AIN76A diet (TestDiet, Richmond, IN) for one week prior to the start of the experiment. They were housed individually in facilities maintained at standard relative humidity and temperature, and 12 h:12 h light:dark conditions, with food and water available *ad libitum*. All procedures involving animals followed NIH guidelines and protocols approved by the Massachusetts Institute of Technology Committee on Animal Care.

Treatment protocol. Treatment groups consisted of 12 male Sprague Dawley rats randomly assigned into four groups of three. Rats were gavaged with corn oil or with 0.7 mmol/kg SF dissolved in corn oil at 30, 32, and 34 days of age (Fig. 2). A previously performed dose–response study in male rats revealed that 0.7 mmol/kg SF was optimal for GST induction (Fiala et al., 2011). Twenty-four hours after the third dose, animals in each group were injected intraperitoneally with 25 μg AFB_1 in DMSO. All animals were euthanized at 4 h or 24 h after AFB_1 administration. This experimental protocol is schematically illustrated in Fig. 2. Livers were collected and submerged in RNA*later* for further gene expression profiling by microarray and for validating the microarray results by RT-PCR.

RNA isolation procedure. Total RNA was isolated from RNA*later*-stabilized rat livers using QIAshredder homogenization and RNeasy® Mini Kit from QIAGEN (Valencia, CA). Total RNA was isolated and purified by a procedure performed as described in QIAGEN's RNeasy® Animal Tissues protocol. The isolation method included a DNase digestion step according to the manufacturer's instructions. Concentration and purity of isolated total RNA were preliminarily verified by checking the absorbance at 260 nm and 280 nm using a DU 730 UV/Vis spectrophotometer (Beckman Coulter, CA, USA). The integrity of total RNA was assessed qualitatively by gel analysis using RNA 6000 Nano chips on an Agilent 2001 (Agilent technologies, Berlin, Germany). RNA samples were used for gene expression analysis by microarray and RT-PCR.

Microarray preparation procedure. All procedures were performed as described in detail in the Affymetrix GeneChip® Expression Analysis Technical Manual. Biotin-labeled cRNA samples for hybridization on GeneChip® Rat 230 2.0 arrays were prepared using the "One-Cycle Target Labeling and Control Reagents complete kit." A set of poly-A controls supplied in the kit was used as a positive control to monitor the entire target labeling process. Total RNA (15 μg) was reverse transcribed using a T7-oligo(dT) Promoter Primer in the first-strand cDNA synthesis reaction. Following this step, the process of RNase H-mediated second-strand cDNA synthesis was performed. The double-stranded cDNA was purified according to the Sample Cleanup Module protocol supplied with the kit and served as a template in the subsequent *in vitro* transcription (IVT) reaction. The IVT reaction was performed in the presence of T7 RNA Polymerase and a biotinylated nucleotide analog/ribonucleotide mix for complementary RNA (cRNA) amplification and biotin labeling. The biotinylated cRNA was spectrophotometrically quantified prior to purification and fragmentation in buffer supplied with the Sample Cleanup Module. Subsequently, the purified fragmented cRNA samples were hybridized to GeneChip® Rat 230 2.0 arrays (Rat 230 2.0) for 16 h at 45 °C in an Affymetrix Hybridization Oven 640. The microarrays were washed and stained with streptavidin–phycoerythrin (SAPE) on an Affymetrix Fluidics Station 450. A signal amplification