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Full Length Research Paper

N-nitrosodimethylamine induced changes in the activities of carcinogen-metabolizing enzymes in the liver of male mice: role of glutathione and gossypol as antioxidants

Sheweita S. A.[1], Mousa N.[1] and Newairy A. A.[2]

[1]Department of Bioscience and Technology, Institute of Graduate Studies & Research,
[2]Department of Biochemistry, Faculty of Science, Alexandria University, Egypt

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The importance of nutrition in protecting living organisms from the toxic effects of environmental carcinogens has attracted the attention of many researchers. In order to exert their toxic and/or carcinogenic effects, most carcinogens need to be activated primarily by phase I drug-metabolizing enzymes including; cytochrome P450, cytochrome b_5, arylhydrocarbon hydroxylase (AHH), N-nitroso-dimethylamine N- demethylase I (NDMA-N-dI), NADPH- cytochrome c reductase and the expression of cytochrome P450 2E1. Changes in the activity of these enzymes were determined in livers of male mice pretreated with either glutathione (GSH) or gossypol as antioxidants, for seven consecutive days before administration of NDMNA as single dose for two hours before decapitation of mice. The total hepatic content of cytochrome P450 and AHH activity were induced, whereas the activity of NDMA-N-dI and expression of cytochrome P450 2E1 were reduced after treatment of mice with NDMA as a single dose. On the other hand, preteatments of mice with either gossypol or GSH for seven consecutive days decreased the total cytochrome P450 content as well as AHH activity whereas the activity of NDMA-N-dI and the expression of cytochrome P450 2E1 increased. Treatment of mice with NDMA only induced the total hepatic content of cytochrome P450 and AHH activity. Interestingly pretreatment of mice with GSH for seven consecutive days before injection of NDMA was found to restore the induced cytochrome P450 and AHH activity caused by NDMA to their normal levels. However, pretreatment of mice with gossypol prior to administration of NDMA did not restore such activities to their normal levels. Treatment of mice with either GSH or gossypol induced the expression of cytochrome P450 2E1. Interestingly, gossypol/NDMA treated mice restored the induced cytochrome P450 2E1 expression, caused by gossypol, to its normal level. However, GSH/NDMA-treated mice did not restore the induced level of cytochrome P450 2E1 caused by GSH, to its normal level. It is concluded that, pretreatment of mice with GSH and gossypol prior to administration of NDMA induced the expression of cytochrome P450 2E1. Therefore, GSH and gossypol, could induce the toxicity and/or carcinogenicity of N-nitrosamines whereas they might protect the liver and probably other organs from the toxic and carcinogenic effects of other carcinogens such as polycyclic aromatic hydrocarbons, e.g. benzo[a]pyrene, through inhibition of total cytochrome P450 content and AHH activity.

Key words: Aryl hydrocarbon hydroxylase, Cytochrome P450 2E1, *N*-nitrosodimethylamine *N*- demethylase, glutathione, antioxidants.

INTRODUCTION

N-nitrosamines are carcinogenic compounds that occur widely in the environment. They can be formed endoge-

*Corresponding author. E-mail: sheweita@hotmail.com.

Abbreviation: Arylhydrocarbon hydroxylase [AHH], *N*-nitrosodi-methylamine-*N*-demethylase, (NDMA-N-dI), *N*-nitrosodimethylamine, [NDMA], benzo[a]pyrene, [B(a)P, cytochrome P450 [CYP450], cytochrome b5 [CYP b5].

nously from the interaction of ingested nitrate or nitrite with secondary amines (Lijinsky and Taylor, 1975) and could be formed in food products. Instances of acute heaptotoxic effects caused by NDMA in ruminants have been recorded (Koppang, 1964) and it is the most volatile nitrosamine present in food samples and beverages (Biaudet et al., 1994). Urinary N -nitrosamines has been reported for populations from widely separate regions of the world and support a link between exposures to this group of chemical carcinogens and the incidence of different types of cancer. Indeed, subjects with high-risks of developing stomach, esophageal, colon and urinary bladder cancers were found to excrete higher levels of N-nitrosamines in their urine compared to their relevant low-risk control groups (Zatonski et al., 1989; Tricker et al., 1989; Mostafa et al., 1994). Their role as causative agents in the carcinogenesis of some human neoplastic diseases has been extensively reviewed (Preussmann, 1984).

N-nitrosamines are activated by NDMA-I and II (NDMA-N-dI and -dII) and cytochrome P450 2E1 in order for them to exert their cytotoxic and carcinogenic effects (Bartsch and Montesano, 1984; Hill, 1988; Arcos et al., 1977). Following the *N*-demethylation by NDMA, a diazonium ion is produced leading ultimately to the formation of carbonium ion which could methylate DNA. It was suggested that the carcinogenic effects of N-nitrosamines are proportional to the activities of their activating enzymes since more of the active metabolites might be produced when NDMA-*N*-demethylase I and cytochrome P450 2E1 are induced (Mostafa and Sheweita, 1992; Sheweita and Mostafa, 1996).

The hepatic cytochrome P450s (CYP) are a multigene family of enzymes that play a critical role in the metabolism of many drugs and carcinogens (Sheweita, 2000). Cytochrome P450 activates polycyclic aromatic hydrocarbons (PAHs) into more reactive intermediates that covalently bind to DNA, a key event in the initiation of carcinogenesis (Jerina et al., 1979; Manchester et al., 1992). An important and extensively studied member of the PAHs is benzo(*a*) pyrene (B(*a*)P), which has been shown to cause cytotoxic, mutagenic and carcinogenic effects in tissues from various animal species (Ashurst and Cohen, 1981; Conney, 1982; Guengerich, 1991). The carcinogenic potency of B(*a*)P is well correlated with the induction of aryl hydrocarbon hydroxylase (AHH) activity and cytochrome P450 1A1 (Conney, 1982; Mannering, 1985). AHH activity can be induced by a wide variety of xenobiotics, drugs, carcinogens, and parasites (Sheweita, 1999) and, hence play an important role in human carcinogenesis (Guengerich, 1991).

The induction of carcinogen- metabolizing enzymes plays a critical step in initiation of carcinogenesis caused by chemical carcinogens such as polycyclic aromatic hydrocarbons and *N*-nitrosamines (Yang et al., 1998; Sheweita, 2000). No previous studies have been conducted on the influence of *N*-nitrosodimethylamine on the activity of carcinogen-metabolizing enzymes as well as the expression of cytochrome P450 2E1 after pretreatment of male mice with glutathione or gossypol, as antioxidants. The present study was planned therefore to investigate the effects of glutathione and gossypol on the cytochrome P450 and cytochrome b_5 contents and the activities of AAH, NDMNA-*N*-dI, of liver before and after treatment of male mice with NDMA.

MATERIALS AND METHODS

Glutathione, glucose-6-phosphate; glucose-6- phosphate dehydrogenase Type XV NDMA, and other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Benzo(*a*)pyrene was obtained from Koch-Light Laboratories, UK.

Animal treatments

Male mice weighing 20 - 25 g were used throughout the study. The local ethics committee approved the design of the experiments, and the protocol conforms to NIH guidelines. Animals were housed, five per cage and given food and water *ad libitum*. Glutathione (100 mg/kg body weight) and gossypol (0.5 mg /kg body weight) were administered to mice orally by gavage once a day for seven consecutive days. NDMA (1 mg/kg body weight) was administered orally also by gavage as a single dose two hours before decapitation to control mice and to both GSH- or gossypol-treated animals. A further control group received distilled water (as vehicle) and assayed with the corresponding treated groups.

Enzyme assays

Liver tissues were homogenization in 3 volumes of 0.1 M of potassium phosphate buffer, pH 7.4. After centrifugation at 12,000 g for 20 min at 4ºC, the supernatant fractions were centrifuged at 105,000 g for 1 h at 4ºC to yield microsomal pellets which were resuspended in 0.1 M potassium phosphate buffer, pH 7.4.

The total microsomal content of cytochrome P450 was determined using the molar extinction coefficient 91 $mM^{-1}Cm^{-1}$ for the reduced cytochrome P-450-carbon monoxide complex and 185 mM^{-1} Cm^{-1} for reduced cytochrome b_5 (Omura and Sato, 1964). Microsomal AHH activity was assayed according to the method of Wiebel and Gelboin (1975); briefly, the incubation mixture (total volume 1ml) contained 50 mM Tris-HCl buffer, pH 7.4, 3 µmole $MgCl_2$, 0.6 µmole NADPH, 100 nmole benzo(a)pyrene, 0.1 ml of microsomal protein (10 mg/ml). The reaction was incubated at 37^oC for 10 min and was terminated by the addition of 1 ml acetone. The reaction mixture was extracted in 2 ml hexane to recover the 3-hydroxy benzo(a)pyrene. The fluorescence intensity of benzo(a)pyrene metabolite measured at excitation and emission wavelengths of 396 and 522 nm respectively.

Microsomal NDMA-*N*--dI activity was assayed according to the method of Venkatesan et al. (1968), with the modification of Mostafa and Sheweita (1992), using a substrate concentration of 4 mM NDMA, which represents the saturation level for this enzyme.

The incubation mixture (total volume was 3 ml) contained 6 µmole $MgCl_2$, 12 µmole niacinamide, 0.4 ml KCl (1.15%), 12 µmole glucose-6-phosphate, 1 unit glucose-6- phosphate dehydrogenase Type XV from Bakers Yeast; 1.2 µmole NADP and 0.25 ml microsomal protein (10 mg/ml) in 0.1 M potassium phosphate buffer pH 7.4. After incubation at 37^oC for 40 min. 1 ml of zinc sulfate (20%) and 1 ml of saturated barium hydroxide were added to terminate the reaction. After centrifugation at 2000 xg, the formaldehyde was

Table 1. Effect of gossypol and Glutathione given as reapeated doses after seven consecutive days on Cytochrome P450, cytochrome b_5, NDMA-*N*-demethylase I, cytochrome c reductase, and aryl hydrocarbon hydroxylase before and after administration of NDMA as single dose for two hours before decapitation of mice.

Parameters	Treatments					
	Control	Glutathione	ol	DMN	Glutathione/DMN	Gossypol/DMN
Total Cytochrome P450	2.5±.012^{b}	1.77±0.21^{b}	0.97±0.11^{c}	8.0±1.23^{a}	2.34±0.13^{b}	6.7±0.89^{a}
Cytochrome b_5	1.1± 0.10^{a}	1.20±0.08^{a}	1.03±0.04^{a}	1.12±0.03^{a}	1.26±0.2^{a}	0.774±0.051^{b}
DMN-demethylase I [NDMA-N-dI]	213.74±18.34^{c}	258.38±14.64^{b}	269.598±16.63^{ab}	109.0±10.1^{d}	310.0±40.6^{a}	280.9±12.67^{ab}
Cytochrome C reductase	0.28±0.01^{b}	0.27±0.01^{b}	0.28±0.02^{b}	0.25±0.01^{b}	0.35±0.03^{a}	0.28±0.01^{b}
Aryl hydrocarbon Hydroxylase [AHH]	22.0±1.49^{a}	13.0±1.9^{b}	10.0±1.9^{b}	37.0±8.6^{c}	23.0±8.2^{a}	22.0 ±1.1^{a}

Parameter contents were expressed as follows: Cytochrome P450 content as nmoles Cyt.P450/mg protein.; Cytochrome b5 content as nmoles Cyt.b5/mg protein; NDMA-dI activity as nmoles HCHO/mg protein/hour and Aryl hydrocarbon hydroxylase (AAH) activity as pmoles 3-OH B(a)P/mg protein/min. Values are the mean ± Standard error of seven mice for each group. Mean values within a row not sharing the same superscript letter were significantly different at P<0.05; those sharing the same superscript letter are significantly different at P<0.05

determined spectrophotometrically from changes in the color intensity of the supernatant at 415 nm. The enzymatic activity of NDMA-*N*-dI was then expressed as nmole of formaldehyde/mg protein/hour. NADPH-cytochrome C reductase activity was assayed by measuring the reducetion of oxidized cytochrome c at 550 nm using an extinctt-ion coefficient of 21 mM^{-1} cm^{-1} (Williams and Kamin, 1962). The protein concentration was measured by the method of Lowry et al. (1951), using bovine serum albumin as standard.

Western blotting

For each sample, 30 µg of liver microsomal protein was prepared and for SDS-polyacrylamide gel electrophoresis. Proteins were electroblotted to Hybond-C membrane (Amersham, UK) and CYP 2E1 isozyme visualized by specific antibodies and detection of chemiluminescence following the manufacturer's instructions (Amersham, UK).

Statistical analysis

Data analyses were conducted using General Linear Model Procedure of Statistical Analysis System (SAS, 1986), and the difference between means was compared using least-squares difference (LSD) at 0.05 significance level.

RESULTS AND DISCUSSION

Antioxidants and total cytochrome P450 content

Nutrition is important in protecting living organisms from the toxic and/or carcinogenic effects of environmental carcinogens through inhibition of their corresponding bioactivating enzymes (Sheweita, 2000; Chessen and Collins, 1997; Sheweita et al., 2001; Polasa et al., 2004). Inhibition of cytochrome P450 system was effective in protecting the liver against the toxicity of a wide variety of toxic agents including carcinogens (Nagabhushan et al., 1987). In keeping with this hypothesis, the generation of toxic metabolites of polycyclic aromatic hydrocarbons, e.g. benzo (a) pyrene, might be decreased since pretreatment of mice with glutathione and/or gossypol as repeated doses was found to decrease the total hepatic content of cytochrome P450 and AHH activity (Table 1).

Gossypol/NDMA-treated mice increased the activity of NADPH- cytochrome c reductase (Table 1). This induction could be one of the animal's defense mechanisms to increase the rate of reduction of cytochrome P450- substrate complex because the total hepatic content of cytochrome P450 decreased in gossypol-treated mice (Table 1). On the other hand, administration of NDMA as a single dose induced the total hepatic content of cytochrome P450 (Table 1). GSH was more effective than gossypol in attenuating the induction of cytochrome P450 caused by NDMA to its normal level (Table 1). The mechanism of protection afforded by the induction of cytochrome P450 by GSH might be due to a stabilization of the sulfhydryl groups (7 in all) of the cysteine residues of cytochrome P450 (Poyer et al., 1978). Pro-tection might result from the antioxidant effects of GSH since induction of cytochrome P450 is associated with generation of free radicals (Valko et al., 2006).

Antioxidants and Aryl Hydrocarbon Hydroxylase [AHH]

As previously reported, activity of AHH depends mainly on the cytochrome P450 content (Sheweita, 2000). Both AHH and cytochrome P450 were induced after a single dose treatment of mice with NDMA (Table 1). NDMA induced the total hepatic

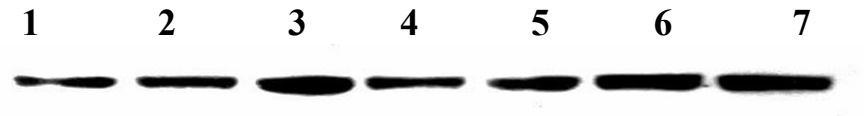

Figure 1. Western blotting analysis against antibodies of Cytochrome P4502E1. All lanes are the pools of 7 samples. Lanes (1 and 2) represent the pooled untreated controls and Lanes (3 - 7) represent the samples from the following treatments; all doses are given as mg/kg body weight to male mice. Lane 3, Gossypol (5 mg) daily for 7 days; Lane 4, NDMA (30 mg) 2 h before sacrifice; Lane 5, Gossypol (5 mg) daily for 7 days followed by a single dose of NDMA(30 mg) 2 h before sacrifice, Lane 6, glutathione (300 mg); Lane 7, glutathione (300 mg) followed by single dose of DMN (30 mg) 2 h before sacrifice.

content of cytochrome P450 and AHH activity (Table 1), therefore, NDMA might induce the toxicity of other carcinogens such as polycyclic aromatic hydrocarbons, e.g. benzo[a]pyrene, which are mainly dependent on total cytochrome P450 and AHH activity, to express their toxic and/or carcinogenic effects. In support of this suggestion, the carcinogenic potency and the extent of binding of benzo[a]pyrene metabolites, epoxides) with DNA and proteins are correlated with the induction of cytochrome P-450-dependent aryl hydrocarbon hydroxylase (Mostafa and Sheweita, 1992; Conney, 1982; Mannering, 1985; Sheweita, 1999).

On the other hand, pretreatment of mice with gossypol or GSH prior to administration of NDMA was found to restore the induced AHH activity to its normal level (Table 1). Return of AHH activity to the pre-induction level could explain the mechanism of protection of these antioxidants against the toxicity and/or carcinogenicity of polycyclic aromatic hydrocarbons, e.g. benzo(a)pyrene.

In agreement with the present results, it has been found that other antioxidants [e.g. diallyl sulfide], exerted an inhibitory effect in colon and renal carcinogenesis in mice and in human epidermal keratinocytes exposed to benzo[a]pyrene (Takahashi et al., 1992; Chun et al., 2004) . Diallyl thioesters, selenium -enriched garlic, and black tea (Marks et al., 1992; Ip et al., 1992; Koul et al., 2005; Halder et al., 2005) have also been reported to reduce the genotoxicity of bone marrow and incidence of mammary tumors in mice caused by benzo(a)pyrene and 7,12-dimethylbenzo(a)anthracene, respectively. A significant reduction in B(a)P-DNA adducts was observed in women given vitamin treatments, suggesting that antioxidant supplementation may mitigate some of the procarcinogenic effects of exposure to B(a)P (Mooney et al., 2005).

Antioxidants and NDMA-N-demethylase I

N-nitroso compounds are potent carcinogens and require metabolic activation by the appropriate enzyme (e.g. NDMA-N-dI) and cytochrome P450 2E1 isozyme in order to exert their cytotoxic and carcinogenic effects (Mostafa and Sheweita, 1992; Sheweita and Mostafa, 1996; Sheweita, 2000). Pretreatment of mice with repeated doses of glutathione or gossypol prior to the administration of NDMA, induced the activity of NDMA-N-dI and also the expression of cytochrome P450 2E1 (Table 1 and Figure 1). Repeated doses of gossypol were more effective than GSH in inducing the activity of NDMA-N-dI (Table 1) and the expression of cytochrome P450 2E1 (Table 1 and Figure 1). Therefore, gossypol could enhance the toxicity of NDMA through induction of its bioactivating enzymes including NDMA-*N*-dI activity and cytochrome P450 2E1 expression. It seems from this study that gossypol and GSH, both of which induced the expression of cytochrome P450 2E1 after NDMA treatment, might also act as protectors against the inhibitory effects of NDMA on these enzymes, since NDMA inhibited both the activity of NDMA-*N*-dI and the expression of cytochrome P450 2E1. Induction of NDMA-*N*-dI and cytochrome P450 2E1 on the other hand might also induce the toxicity and carcinogenicity of N -nitrosamines. Supporting this suggestion, it has been found that Inhibition of cytochrome P450 2E1 and NDMA-*N*-dI activity could play a significant role in the reduction of tumorigenicity and carcinogenicity of *N*-nitrosamines. Moreover, it has been shown that the administration of diallylsulfide [DAS], the active ingredient of garlic which inhibit NDMA-N -dI activity, was found to decrease the tumorgenicity and the level of the DNA adduct (*O*6-methyldeoxyguanosine) in different organs of mice pretreated with *N*-nitrosomethylbenzylamine (Ludeke et al., 1992) and also the level of *N*7-, *O*6-methyldeoxyguanosine DNA adducts in mammary and liver tissues of mice given *N*-methyl-*N*-nitrosourea or *N*-nitrosodimethylamine (Lin et al., 1994).

In conclusion, pretreatment of mice with GSH and gossypol prior to administration of NDMA induced the expression of cytochrome P450 2E1. This induction might explain the mechanism of toxicity and/or carcinogenicity of *N*-nitrosamines since these compounds are dependent on cytochrome P450 2E1 expression and NDMN-N-dI activity in order to exert their carcinogenic effects. On the other hand, gossypol and GSH could protect the liver and probably other organs from the toxic and carcinogenic effects of other carcinogens, e.g., benzo[a]pyrene, through inhibition of total cytochrome P450 content.

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