Nordihydroguaiaretic acid is a potent inhibitor of ferric-nitrilotriacetate-mediated hepatic and renal toxicity, and renal tumour promotion, in mice

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Ferric-nitrilotriacetate (Fe-NTA) is a known renal carcinogen. In the present study, we report the effect of a potent lignin-derived herbal antioxidant, nordihydroguaiaretic acid (NDGA), against Fe-NTA-mediated tissue toxicity. Fe-NTA (alone) treatment of mice enhances ornithine decarboxylase activity to 259% in liver and 341% in kidney and increases [3H]thymidine incorporation in DNA to 250% in liver and 324% in kidney compared with the corresponding saline-treated controls. The enhanced ornithine decarboxylase activity and DNA synthesis showed a reduction to 138 and 123%, respectively, in liver at a higher dose of 2 mg NDGA/day/animal whereas in kidney the reduction was to 118 and 102%, respectively, compared with the corresponding saline-treated controls. In the Fe-NTA (alone)-treated group, a 12% renal tumour incidence was recorded whereas, in N-diethylthiourea (DEN)-initiated and Fe-NTA-promoted animals, the percentage tumour incidence was increased to 68% as compared with untreated controls. No tumour incidence was recorded in the DEN-initiated, non-promoted group. The administration of NDGA, afforded >80% protection against DEN- and Fe-NTA-mediated renal tissue injury in vivo. Fe-NTA treatment also enhanced hepatic and renal microsomal lipid peroxidation to 170 and 205% of saline-treated controls, respectively, and hydrogen peroxide generation by 2.5-fold in both tissues accompanied by a 51 and 21% decrease in the level of glutathione and 35–48 and 35–50% decrease in the activities of glutathione-metabolizing and antioxidant enzymes in liver and kidney, respectively. These changes were reversed significantly in animals receiving a pretreatment of NDGA. Our data show that NDGA can abrogate the toxic and tumour-promoting effects of Fe-NTA in liver and kidney of mice and can serve as a potent chemopreventive agent to suppress oxidant-induced tissue injury and tumorigenesis.

Introduction

In recent years, attention has been focused on the identification of naturally occurring plant phenols as possible cancer chemopreventive agents (1,2). Several studies have shown that certain naturally occurring plant phenols possess substantial antimutagenic and anticarcinogenic effects against a variety of polycyclic aromatic hydrocarbons (3). The mechanism by which naturally occurring plant phenols exert their antimutagenic and anticarcinogenic effects is believed to be the result of one or more of the events, including the interaction with the cytochrome P-450 activation system, which lead to reduced formation of ultimate carcinogenic metabolite and/or scavenging of the reactive molecular species of carcinogenic metabolites to prevent their interaction with the critical sites (1). In addition, antioxidant properties of some of these agents help them to combat the excessively generated oxidants by the pro-oxidant carcinogenic agents, and thereby diminish tissue injury.

Ferric-nitrilotriacetate (Fe-NTA) is one such molecule, which causes hepatic and renal damage by increasing oxidative stress. Our laboratory studies have shown that Fe-NTA is a hepatic tumour promoter and it depletes glutathione (GSH) levels and causes a decrease in the activities of GSH-metabolizing and antioxidant enzymes in liver (4,5). Fe-NTA is also a known potent nephrotoxic agent and induces apoptosis in mouse renal proximal tubules (6). Renal toxicity is assumed to be caused by the elevation of serum-free iron concentration, following its reduction at the luminal side of the proximal tubule, which generates reactive oxygen species (ROS) and leads to the enhancement in lipid peroxidation (7). Recently, an enhanced formation of 4-hydroxy-2-nonenal-modified proteins in the renal proximal tubules of rats treated with Fe-NTA has been shown (8). Repeated i.p. administration produces acute and subacute proximal tubular necrosis in kidney (9), which is subsequently associated with a high incidence of renal adenocarcinoma in male mice and rats (9,10). It is assumed that the Fe-NTA-mediated generation of free radicals plays an important role in renal tumorigenesis. Renal DNA damage leading to single strand and double strand DNA breaks (11), DNA–protein cross links (12) and enhanced formation of 8-hydroxy deoxyguanosine (8-OH-dG) has been observed following exposure of animals to Fe-NTA (13). It has been shown that Fe-NTA stimulates OH– production, which is responsible for initiating many of these effects (14).

We therefore hypothesize that a potent antioxidant may suppress its toxicity if given as a pre-treatment to animals receiving Fe-NTA. Recently, we have shown that the pre-treatment with vitamin E, a membrane-bound antioxidant, reduces the toxicity of Fe-NTA (15). Another synthetic lipophilic antioxidant, probucol, has also been found effective in reducing Fe-NTA-mediated kidney damage (16). These studies prompted us to search for a novel and effective agent that may abrogate the toxicity and carcinogenicity of Fe-NTA. Among the known dietary and herbal agents that have the desired antioxidant properties, nordihydroguaiaretic acid (NDGA) is a typical and simple lignin present in evergreen shrubs such as Larrea divarita, which is found all over the world, and has 12% of the active ingredient in the dry plant. NDGA is a potent inhibitor of the lipoxygenase activity (17) besides being

Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; DEN, N-diethylthiourea; DTNB, 5,5'-dithio-bis-2-nitrobenzoic acid; Fe-NTA, ferric-nitrilotriacetate; GSH, glutathione; NDGA, nordihydroguaiaretic acid; ODC, ornithine decarboxylase; 8-OH-dG, 8-hydroxy deoxyguanosine; PCA, perchloric acid; PMS, post-mitochondrial supernatant; PMSF, phenylmethylsulphonyl fluoride; RCT, renal cell tumour; ROS, reactive oxygen species; TBA, thiobarbituric acid; TCA, trichloroacetic acid.
a very strong antioxidant molecule. NDGA is traditionally used as an antioxidant in animal fat, vegetable oil and milk products in US food industries. NDGA protects against TPA-induced tumour promotion in mouse skin (18) and TPA-caused induction of ornithine decarboxylase (ODC) (19) and protein kinase C (20) NDGA also inhibits benzyl peroxide-mediated epidermal ODC induction and skin tumour promotion in Sencar mice (21). Since NDGA has been shown to inhibit various kinds of injuries and neoplasm, it is possible that pre-treatment of animals with NDGA may protect against Fe-NTA mediated hepatic and renal injury. In this study, we show the effect of prophylactic treatment of NDGA on mice receiving Fe-NTA treatment. Our results show that NDGA can diminish Fe-NTA-mediated oxidative injury, suppress hyperproliferative response and lessen hepatic and renal toxicity. In addition, NDGA also reduces the renal tumour promotion response to Fe-NTA in N-diethylntosamine (DEN)-initiated mice. Our data indicate that NDGA may act as a potential hepatic and renal protective agent.

Materials and methods

Chemicals

NDGA, DEN, horseradish peroxidase, phenol red, Tris–HCl, thiobarbituric acid, oxidized and reduced GSH, NADPH, 2-mercaptoethanol, dithiothreitol, phenylmethylsulphonyl fluoride (PMSF), pyridoxal-5-phosphate, glucose-6-phosphate, 1-chloro-2,4-dinitrobenzene (CDNB), γ-glutamyl-p-nitroanilide, 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) and nitroliotriacetic acid (NTA) were purchased from Sigma (St Louis, MO). [14C]ornithine (sp. act. 56 m Ci/mmol) and [3H]thymidine (sp. act. 82 Ci/mmol) were purchased from Amersham (Buckinghamshire, UK). All other chemicals and reagents used were of the highest commercially available purity.

Preparation of Fe-NTA

The Fe-NTA solution was prepared by the method of Awai et al. (22).

Animals and treatment

Male Swiss albino mice (4–6 weeks old) weighing 20–25 g, from Jamia Hamdard, Central Animal House colony, were used throughout this study. Animals were housed in an air-conditioned room and had free access to a pellet diet (Hindustan Lever, Bombay, India).

Different groups of animals were used for the various sets of biochemical studies. For studying the effect of NDGA on Fe-NTA-mediated generation of hepatic and renal oxidative stress and ODC induction, 30 male mice weighing 20–25 g were divided into six groups of six mice per group. Group I received saline and served as negative control. Group II received an oral treatment of NDGA (only higher dose) and served as a control. Groups IV and V received NDGA at dose levels of 1 and 2 mg/animal/day in 0.2 ml corn oil, respectively, for a period of 1 week, administered by gavage. Group III received only corn oil (the vehicle of NDGA) for a period of 1 week by gavage. At 24 h after the last treatment of NDGA or corn oil the animals from groups III, IV and V received an i.p. injection of Fe-NTA (9 mg Fe/kg body wt). All the 30 animals were killed by cervical dislocation within a period of 1 h, at 12 h after treatment with Fe-NTA or saline. Just before killing, blood of these animals was collected in different test-tubes from the jugular vein. Their liver and kidneys were quickly removed, cleaned free of extraneous material and homogenized in distilled water for further processing and the separation of DNA.

For studying [3H]thymidine incorporation into hepatic and renal DNA, animal treatment protocol and dose regimens were the same as described above. However, at 18 h after treatment with Fe-NTA, these animals were given an i.p. injection of [3H]thymidine (20 µCi/animal). Two hours after administration of [3H]thymidine, these animals were killed by cervical dislocation. Their liver and kidneys were quickly removed, cleaned free of extraneous material and homogenized in distilled water for further processing and the separation of DNA.

For tumorigenesis studies using the initiation–promotion protocol, the animals were divided into six groups of 25 mice/group. Group I received saline and served as negative control and the animals in groups II, IV and VI were initially treated with a single i.p. injection of DEN at a dose level of 100 mg/kg body wt in saline. Ten days after the initiation, the animals in groups IV, VI and V were treated with increasing doses of Fe-NTA (1.0–6 mg Fe/kg body wt, in Fe-NTA) administered i.p., 3 days a week for 16 weeks. Groups V and VI received an oral treatment of NDGA at a dose level of 1 and 2 mg/animal/day in 0.2 ml corn oil, respectively, for a period of 16 weeks, three times a week. At the end of 24 weeks, the animals were killed by cervical dislocation and their livers and kidneys were quickly removed and preserved in 10% neutral buffered formalin for histopathological studies. Haematoxylin and eosin preparations of processed sections were prepared for microscopic examination.

Post-mitochondrial supernatant (PMS) and microsome preparation

Animals and treatment

Kiddys and livers were quickly removed, perfused immediately with ice-cold saline (0.85% w/v NaCl) and homogenized in chilled phosphate buffer (0.1 M, pH 7.4) that contained KCl (1.17% w/v), using a Potter Elvehjem homogenizer. The homogenate was filtered through a muslin cloth and was centrifuged at 800 g for 5 min at 4°C in an Eltek Refrigerated Centrifuge (model RC 4100 D) to separate the nuclear debris. The aliquot obtained was centrifuged at 10 500 g for 20 min at 4°C to obtain PMS, which was used as a source of enzymes. A portion of the PMS was centrifuged in an ultracentrifuge (Beckman, L7-55) at 100 000 g for 60 min at 4°C. This pellet was considered to be the microsomal fraction and was suspended in phosphate buffer (0.1 M, pH 7.4) containing KCl (1.17% w/v).

Estimation of reduced GSH

Reduced GSH in kidney and liver was assayed by the method of Jollow et al. (23). An aliquot of 1.0 ml of renal and hepatic PMS (10% w/v) was precipitated with 1.0 ml of sulphasalicylic acid (4% w/v). The samples were kept at 4°C for at least 1 h and then subjected to centrifugation at 1200 g for 15 min at 4°C. The assay mixture contained 0.1 ml filtered aliquot, 2.7 ml phosphate buffer (0.1 M, pH 7.4) and 0.2 ml DTNB (40 mg/10 ml of phosphate buffer 0.1 M, pH 7.4) in a total volume of 3.0 ml. The yellow colour developed was read immediately at 412 nm on a spectrophotometer (Milton Roy Model-21D).

Lipid peroxidation

The assay for renal and hepatic microsomal lipid peroxidation was done following the method of Wright et al. (24). The reaction mixture, in a total volume of 1.0 ml, contained 0.58 ml phosphate buffer (0.1 M, pH 7.4), 0.2 ml of renal and hepatic microsome (10% w/v), 0.2 ml ascorbic acid (100 mM) and 0.02 ml ferric chloride (100 mM). The reaction mixture was incubated at 37°C in a shaking water bath for 1 h. The reaction was stopped by the addition of 1.0 ml trichloroacetic acid (TCA) (10% w/v). Following addition of 1.0 ml thiobarbituric acid (TBA) (0.67% w/v), all the tubes were placed in a boiling water bath for a period of 20 min. At the end, the tubes were shifted to an ice-bath and centrifuged at 2500 g for 10 min. The amount of malonaldehyde formed in each of the samples was assessed by measuring the optical density of the supernatant at 535 nm using a spectrophotometer against a reagent blank. The results were expressed as nmol MDA formed/kg tissue at 37°C using a molar extinction coefficient of 1.56×105 M/cm.

DNA synthesis

The incorporation of [3H]thymidine in renal and hepatic DNA was done by the method of Smart et al. (25) as described by Iqbal et al. (4). The kidneys and livers were quickly removed, cleaned free of extraneous material, and the homogenate (10% w/v) was prepared in ice-cold water. The precipitate obtained was washed with cold TCA (5% w/v) and incubated with cold perchloric acid (PCA) (10%) at 4°C overnight. After incubation it was centrifuged and the precipitate was washed with cold PCA (5%). The precipitate was dissolved in warm PCA (10% w/v) followed by incubation in a boiling water-bath for 30 min, and then filtered through Whatman paper. The filtrate was counted for 3H in a liquid scintillation counter (LKB-Wallace-1410). The amount of DNA in the filtrate was estimated by the diphenylamine method of Giles and Myers (26). The amount of [3H]thymidine incorporated was expressed as d.p.m./µg DNA.

ODC activity

ODC activity was determined by utilizing 0.4 ml of renal and hepatic 105 000 g post-mitochondrial supernatant per ml of tube and measuring the release of 14CO2 from nS-[1-14C]ornithine by the method of O’Brien et al. (27), as described by Athar et al. (28). The kidneys and livers were homogenized in Tris–HCl buffer (pH 7.5, 50 mM) that contained EDTA (0.1 mM), pyridoxal phosphate (0.1 mM), PMSF (1.0 mM), 2-mercaptoethanol (1.0 mM), dithiothreitol (0.1 mM) and Tween 80 (0.1% w/v), at 4°C using a polytron homogenizer (Kinetamica AGP 3000). In brief, the reaction mixture contained 400 µl of post-mitochondrial supernatant that contained pyridoxal phosphate (0.32 mM), EDTA (0.4 mM), dithiothreitol (4.0 mM), ornithine (0.4 mM), brig 35 (0.02% w/v) and [14C]ornithine (0.05 µCi) in a total volume of 0.495 ml. After adding buffer and the co-factor mixture to a blank and another test tube, the tubes were covered immediately with a rubber cork containing 0.2 ml ethanolamine and methoxyethanolamine mixture in the central well, and with 0.1 ml water-bath at 37°C. After 1 h of incubation, the enzymatic activity was arrested by injecting 1.0 ml of citric acid solution (2.0 M) along the sides of glass tubes and the incubation was continued for 1 h to ensure complete absorption of 14CO2. Finally, the central well was transferred to a vial.
 containing 2 ml of ethanol and 10 ml toluene-based scintillation fluid was added to it, followed by counting the radioactivity in a liquid scintillation counter. ODC activity was expressed as pmol $^{14}$CO$_2$ released/h/mg protein.

**Glucose 6-phosphate dehydrogenase activity**

The activity of glucose 6-phosphate dehydrogenase was assayed by the method of Zaheer et al. (29). The reaction mixture in a total volume of 3.0 ml consisted of 0.3 ml Tris–HCl buffer (0.05 M, pH 7.6), 0.1 ml NADP (0.1 mM), 0.1 ml glucose 6-phosphate (0.8 mM), 0.1 ml MgCl$_2$ (8 mM), 0.3 ml of renal and hepatic PMS (10% w/v) and 2.1 ml distilled water. The changes in absorbance were recorded at 340 nm and the enzyme activity was calculated as nmol NADP reduced/min/mg protein using a molar extinction coefficient of $3.14 \times 10^3$ M/cm.

**Glutathione reductase activity**

Glutathione reductase activity was assayed by the method of Carlberg and Mannervik (30), as modified by Mohandas et al. (31). The assay system consisted of 1.65 ml phosphate buffer (0.1 M, pH 7.6), 0.1 ml EDTA (0.5 mM), 0.05 ml oxidized glutathione (1 mM), 0.1 ml NADPH (0.1 mM) and 0.1 ml of renal and hepatic PMS (10% w/v) in a total volume of 2.0 ml. The enzyme activity was quantitated at 25°C by measuring the disappearance of NADPH at 340 nm, and was calculated as nmol NADPH oxidized/min/mg protein using a molar extinction coefficient of $6.22 \times 10^3$ M/cm.

**Catalase activity**

Catalase activity was assayed by the method of Clai-born (32). Briefly, the assay mixture consisted of 1.95 ml phosphate buffer (0.05 M, pH 7.0), 1.0 ml hydrogen peroxide (0.019 M), 0.05 ml and 0.025 ml of renal and hepatic PMS (10% w/v), respectively, in a final volume of 3.0 ml. Changes in absorbance were recorded at 240 nm. Catalase activity was calculated in terms of nmol H$_2$O$_2$ consumed/min/mg of protein.

**Glutathione S-transferase activity**

Glutathione S-transferase activity was measured by the method of Habig et al. (33), as described by Athar et al. (34). The reaction mixture consisted of 1.425 ml phosphate buffer (0.1 M, pH 6.5), 0.2 ml reduced glutathione (1 mM), 0.025 ml CDNB (1 mM), 0.3 ml and 0.05 ml of renal and hepatic PMS (10% w/v), respectively, in a total volume of 2.0 ml. The changes in absorbance were recorded at 340 nm and enzyme activity was calculated as nmol CDNB conjugated formed/min/mg protein using a molar extinction coefficient of 9.6 $\times 10^3$ M/cm.

**Hydrogen peroxide (H$_2$O$_2$) assay**

H$_2$O$_2$ was assessed by H$_2$O$_2$-mediated horseradish peroxidase-dependent oxidation of phenol red by the method of Pick and Keisari (35), with a slight modification. Renal and hepatic microsomes 2.0 ml (10% w/v) of each were suspended in 1.0 ml of solution containing phenol red (0.28 mM), horseradish peroxidase (8.5 U), dextrose (5.5 mM) and phosphate buffer (0.05 M, pH 7.0) and were incubated at 37°C for 60 min. The reaction was stopped by addition of 0.01 ml NaOH (10 N) and then centrifuged at 800 g for 5 min. The absorbance of the supernatant was recorded at 101 nm against a reagent blank. The quantity of H$_2$O$_2$ produced was expressed as nmol H$_2$O$_2$/g tissue based on the standard curve of H$_2$O$_2$-oxidized phenol red.

**Estimation of creatinine**

Creatinine was estimated by the alkaline picrate method of Hare (36). Protein-free filtrate was prepared. To a 1.0 ml of plasma/serum, 1.0 ml of sodium tungstate (5% w/v), 1.0 ml of sulphuric acid (0.6 N) and 1.0 ml of distilled water were added and mixed thoroughly, and centrifuged at 800 g for 5 min. The supernatant was added to a mixture containing 1.0 ml of picric acid (1.05% w/v) and 1.0 ml of sodium hydroxide (0.75% w/v). The absorbance was recorded exactly after 20 min at 520 nm.

**Estimation of blood urea nitrogen**

Blood urea nitrogen was estimated by dia-cetyl monoxime method of Kanter (37). Protein-free filtrate was prepared. To a 0.5 ml of protein-free filtrate, 3.5 ml of distilled water, 0.8 ml diacetylmonoxime (2%) and 3.2 ml of sulphuric acid–phosphoric acid reagent (reagent was prepared by mixing 150 ml of 85% w/v phosphoric acid with 140 ml of water and 50 ml of concentrated H$_2$SO$_4$) was added. The reaction mixture was placed in a boiling water-bath for 30 min and then cooled. The absorbance was recorded at 450 nm.

**γ-Glutamyl transpeptidase activity**

The γ-glutamyl transpeptidase activity was determined by the method of Orlowski and Meister (38) using γ-glutamyl-p-nitroanilide as a substrate. The reaction mixture was in a total volume of 1.0 ml and contained 0.2 ml homogenate (10%), which was incubated with 0.8 ml of the substrate mixture (containing 4 mM γ-glutamyl-p-nitroanilide, 40 mM glycylglycine and 11 mM MgCl$_2$ in 185 mM Tris–HCl buffer, pH 8.25) at 37°C. Ten minutes after the initiation of the reaction, 1.0 ml of TCA (25%) was added and mixed to terminate the reaction. The solution was centrifuged and the supernatant fraction was read at 405 nm. The enzyme activity was calculated as nmol γ-glutamyl-p-nitroaniline formed/min/mg protein using a molar extinction coefficient of $p$-nitroanilone of 1.74 $\times 10^3$ M/cm.

**Results**

**Effect of pre-treatment of mice with NDGA on Fe-NTA-mediated induction of hepatic and renal ODC activity**

The Fe-NTA (alone) treatment resulted in ~259% induction of ODC activity in liver and 341% in kidney as compared with their respective saline-treated control (Figure 1). However, pre-treatment of mice with NDGA reduced induction of ODC in both organs. A lower dose of 1 mg/animal/day of NDGA marginally reduced the Fe-NTA-mediated ODC induction to ~197% in liver and 167% in kidney compared with saline-treated controls, but at a higher dose of 2 mg/animal/day it was reduced to ~138 and 118% compared with the saline-treated controls.

**Effect of pre-treatment of mice with NDGA on Fe-NTA-mediated enhancement of hepatic and renal [3H]thymidine incorporation**

The Fe-NTA (alone) treatment resulted in an ~2.5-fold enhancement of the incorporation of [3H]thymidine in hepatic DNA and an ~3-fold increase in renal DNA (Figure 2). However, in NDGA pre-treated animals, this enhancement was significantly less compared with the Fe-NTA (alone)-treated group. The decrease in [3H]thymidine incorporation was dependent on the
dose of NDGA used, as has been observed with the ODC induction studies.

Effect of pre-treatment of mice with NDGA on the Fe-NTA-mediated enhancement of the value of serum creatinine and blood urea nitrogen

The Fe-NTA (alone) treatment led to an enhancement of ~2.5- and 2.8-fold in the values of serum creatinine and blood urea nitrogen, respectively (Figure 3). Administration of Fe-NTA to mice that were receiving a pre-treatment of NDGA led to a significant diminution in both of these values. The effect was dose-dependent, though at both doses of NDGA a significant reduction in the values of blood urea nitrogen and serum creatinine could be observed.

Effect of pre-treatment of DEN-initiated mice with NDGA on Fe-NTA-mediated renal tumour promotion

Chronic treatment of Fe-NTA to DEN-initiated animals led to high mortality, which meant that the tumorigenesis experiment could not be continued beyond 24 weeks. Most of the animals died as a result of nephropathy. The data given in Table I give a summary of the percentage incidence of renal cell tumours (RCTs) in different treatment groups of saline and Fe-NTA-treated animals. Saline (alone)- or DEN (alone)-treated groups did not show any tumours. However, treatment with Fe-NTA of the DEN-initiated animals enhanced the development of RCTs in ~68% of the animals studied whereas treatment with Fe-NTA of the uninitiated animals led to the development of RCTs in ~12% of the animals studied. In comparison, tumour incidence in the group of animals pre-treated with NDGA, at a lower dose of 1 mg/animal/day was 20% whereas in the group receiving the higher dose of NDGA (2 mg/animal/day) the tumour incidence was reduced to almost 16%.

The histopathology of the renal tumour tissue, which was initiated with DEN and promoted with Fe-NTA, and its protection with NDGA, is shown in Figure 4. The tissue sections from kidney of mice treated with DEN and Fe-NTA, either with or without pre-treatment of NDGA, were examined for the degree of infiltration of leucocytes, tumour cells and hyperchromatism. In the group treated with DEN (alone), no particular biochemical or histological changes were observed (data not shown). The sections from the Fe-NTA (alone)-treated group showed marked inflammatory leucocytic infiltrate and interstitial connective tissue infection, which were absent in the saline (alone)-treated control group. However, the DEN-initiated and Fe-NTA promoted group showed a focal collection of leucocytic infiltratory cells and adenocarcinomas with hyperchromatism and enlargement of nuclei in the tubular epithelium. Most of these changes were significantly alleviated in mice pre-treated with NDGA at both dose levels (1 and 2 mg/animal/day), which was comparable with the untreated controls.

The histopathology of the liver tissue, initiated with DEN and promoted with Fe-NTA, and their protection with NDGA, is shown in Figure 5. Sections from the Fe-NTA (alone)-treated group showed hyperplastic lesions; however, the intensity of such changes was minimal. On the contrary, histological sections from liver of mice treated with DEN and Fe-NTA showed a mild hyaline cast and marked hyperplasia with extensive degeneration of cells. However, NDGA pre-treatment to mice at both dose levels suppressed these effects and the sections were not visually different from those of untreated controls under the microscope. Livers from DEN-initiated animals showed normal histology.
NDGA is a potent inhibitor of Fe-NTA-mediated toxicity

Table I. Summary of tumour data of the effect of NDGA on DEN- and Fe-NTA-induced renal tumours

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>No. animals treated histopathologically</th>
<th>No. animals studied with renal cell tumours</th>
<th>No. animals of tumours</th>
<th>% incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Saline (alone)</td>
<td>25</td>
<td>20</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>2. DEN (alone)</td>
<td>25</td>
<td>21</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>3. DEN + Fe-NTA</td>
<td>25</td>
<td>19</td>
<td>13</td>
<td>68</td>
</tr>
<tr>
<td>4. Fe-NTA (alone)</td>
<td>25</td>
<td>20</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>5. NDGA(D1) + DEN + Fe-NTA</td>
<td>25</td>
<td>21</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>6. NDGA(D2) + DEN + Fe-NTA</td>
<td>25</td>
<td>20</td>
<td>4</td>
<td>16</td>
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</table>

Fig. 4. Histopathological sections of kidney of (A) normal untreated, (B) Fe-NTA-treated, (C and D) DEN and Fe-NTA-treated, (E and F) DEN, Fe-NTA-treated and NDGA (doses 1 and 2) pre-treated animals. Specimens stained with haematoxylin and eosin. Other details are provided in the text. (A, B, D–F) ×200, (C) ×100.

Effect of pre-treatment of mice with NDGA on the Fe-NTA-mediated enhancement of lipid peroxidation and the generation of \( \text{H}_2\text{O}_2 \)

The Fe-NTA (alone) treatment enhanced hepatic and renal microsomal lipid peroxidation to ~170 and 205% compared with the saline-treated controls, respectively (Table II), whereas there was an enhancement in \( \text{H}_2\text{O}_2 \) generation of >2.5-fold in both liver and kidney. However, NDGA pre-treatment dose-dependently inhibited the enhancement of Fe-NTA-mediated lipid peroxidation and \( \text{H}_2\text{O}_2 \) generation. Although, there is a significant reduction in oxidant generation with both the doses of NDGA studied, with the higher dose of NDGA (2 mg/animal/day) the level of lipid peroxidation was similar to the value of control group.

Fig. 5. Histopathological sections of liver of (A) normal untreated, (B) Fe-NTA-treated, (C and D) DEN and Fe-NTA-treated, (E and F) DEN, Fe-NTA-treated and NDGA (doses 1 and 2) pre-treated animals. Specimens stained with haematoxylin and eosin. Other details are provided in the text. (A, B, D–F) ×200, (C) ×400.

Effect of pre-treatment of mice with NDGA on Fe-NTA-mediated renal and hepatic levels of GSH, and GSH-metabolizing and antioxidant enzymes

The Fe-NTA (alone) treatment diminished the levels of GSH, glutathione S-transferase, glutathione reductase, glucose 6-phosphate dehydrogenase and catalase to 51, 41, 43, 48 and 35% in liver and to 21, 50, 29, 34 and 35% in kidney, respectively, compared with their corresponding saline-treated control values (Figures 6 and 7). Treatment of mice with NDGA prior to treatment with Fe-NTA resulted in the recovery of reduced levels of GSH, GSH-metabolizing enzymes and antioxidant enzymes. In liver the recovery ranged from 32 to 42% whereas in kidney it ranged from 42 to 54% with the lower dose of NDGA. At the higher dose level, the recovery ranged from 49 to 51% in liver and 47 to 59% in kidney.
Table II. Effect of NDGA on Fe-NTA-induced hepatic and renal microsomal lipid peroxidation and generation of H$_2$O$_2$

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Lipid peroxidation</th>
<th>H$_2$O$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of control</td>
<td>% of control</td>
</tr>
<tr>
<td></td>
<td>(liver)</td>
<td>(kidney)</td>
</tr>
<tr>
<td>Saline</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>NDGA (D$_2$)</td>
<td>101</td>
<td>106</td>
</tr>
<tr>
<td>Fe-NTA</td>
<td>170*</td>
<td>205*</td>
</tr>
<tr>
<td>NDGA (D$_1$)</td>
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</tr>
<tr>
<td>+ Fe-NTA</td>
<td>116**</td>
<td>134**</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SE of six animals. Saline-treated animals served as controls.

*Significantly different (P < 0.05) when compared with saline-treated group.

**Significantly different (P < 0.001) when compared with Fe-NTA-treated group.

D$_1$ and D$_2$ represent doses 1 and 2; administration of 1 or 2 mg NDGA/animal/day, given orally 1 week before the treatment with Fe-NTA.

Discussion

A wide range of studies have shown that several naturally occurring phenols possess significant antitumour-promoting activity because of their antioxidant properties (40,41). It has been shown that NDGA possesses polar but non-ionic groups, interacts with a variety of high affinity sites of enzyme proteins, such as Cyt P-450. It thereby inhibits monooxygenase activity respectively, compared with their saline-treated controls. However, hepatic and renal γ-GGT were increased by 186 and 184% compared with the untreated controls, and was reduced significantly by treatment with NDGA.
and provides a possible mechanism for the protective role of NDGA against the onset of carcinogenesis, which is induced by the chemicals that are metabolized by the cytochrome P-450-dependent enzyme system. Recently, we have demonstrated that Fe-NTA, a known complete carcinogen, is a potent renal tumour promoter and promotes DEN-initiated renal tumorigenesis (42). However, it does not require the cytochrome P-450 system to manifest its effect. Hence, the observed inhibition by NDGA of the DEN-initiated and Fe-NTA-promoted renal carcinogenesis in the present study could be caused by its known effect to act as a strong antioxidant. However, in liver there was comparatively less damage to the DEN-initiated and Fe-NTA-promoted group, though, a significant recovery could be observed in NDGA-pre-treated groups. Similarly, the chemopreventive effects of N-(4-hydroxyphenyl) retinamide against the exogenous and endogenous development of putative preneoplastic, glutathione S-transferase placental form-positive lesions in rat liver have also been shown (43). Recently, acetylsalicylic acid, a cyclooxygenase inhibitor has been shown to inhibit foci development as well as 8-OH-DG formation in the liver, and also inhibits diet-associated induction of liver cell carcinoma (44). In support of our earlier reported data of oxidant-mediated damage in Fe-NTA-treated animals, the observed inhibition of renal and hepatic damage by NDGA indicates that Fe-NTA produces hepatic and renal damage largely through the generation of oxidative stress. In a similar study, we observed that garlic oil protects renal injuries caused by Fe-NTA (45), though the mechanism of inhibition may be different. Induction of ODC activity and enhancement of [3H]thymidine incorporation are extensively used as biochemical markers to evaluate the tumour-promoting potential of an agent (46). Based on this information, we assessed the effect of NDGA on Fe-NTA-mediated induction of biochemical responses of tumour promotion. The observed suppression in the Fe-NTA-mediated enhancement of ODC activity and [3H]thymidine incorporation in both liver and kidney by the pre-treatment of mice with NDGA in the present study suggest its ability to act as a potent antitumour promoter in the two organs. The inhibition of Fe-NTA-induced hepatic and renal ODC activity by NDGA in the present study is parallel to the inhibition in cutaneous ODC activity, as observed by Nakadate et al. (19). There appears to be a good correlation between the antitumour promoting activity of various antioxidants and their ability to inhibit ODC induction in mouse skin (47). These studies also corroborate our previous observations in skin where NDGA was observed to diminish the tumour-promoting effect of the oxidant tumour promoter, benzoyl peroxide (21).

Thus, it appears that NDGA, because of its antioxidant potential, triturates the oxidant metabolites generated by these toxic compounds. This is further evidenced by the reduction in the Fe-NTA-mediated enhanced hepatic and renal lipid peroxidation and H2O2 generation. Also, an increase in activity of γ-GGT may lead to the accumulation of glycine and cysteine, thereby reducing Fe-NTA to its ferrous complex and enhancing peroxidative damage to the membrane or tissue. The reversal in enzymatic and non-enzymatic antioxidant molecules in the present study supports the theory that the action of NDGA is beyond its direct interaction with the excessively generated oxidants. It seems that NDGA acts at several loci in the metabolic path to prevent Fe-NTA-induced injury, thus suggesting that such treatment could protect the cells/tissues against cytotoxic/genotoxic effects of peroxides and OH generated from Fe-NTA.

Our results, which show inhibition of Fe-NTA-induced hepatic and renal toxicity by NDGA, may also have been caused by the antioxidant and free radical scavenging action of NDGA. By scavenging free radicals and inhibiting ODC induction and DNA synthesis, NDGA may intercept the growth-promoting and mutagenic functions of polyamines and arachidonic acid metabolites. Furthermore, the influence of NDGA on the action of Fe-NTA-mediated tumour promotion in DEN-initiated mice is of interest. Both doses of NDGA used in the present study not only reduced organ-specific toxicity caused by Fe-NTA but also showed its anticarcinogenic efficacy by suppressing tissue carcinogenesis via inhibition of tumour formation.

In summary, our data indicate that the naturally occurring antioxidant NDGA can alleviate Fe-NTA-mediated nephro- and hepatotoxicity and tumour promotion. The precise mechanism(s) of inhibitory effects of NDGA are still incompletely understood; however, it may involve inhibition of excessive oxidant formation and interaction of electrophilic species, including free radicals with macromolecules. Because NDGA is non-toxic and utilized as a food supplement, it could also prove useful in diminishing oxidant-induced hepatic and renal injuries in humans.

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