Resveratrol Inhibits N-nitrosodiethylamine-Induced Ornithine Decarboxylase and Cyclooxygenase in Mice

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Summary

Increased levels or overexpression of ornithine decarboxylase (ODC), a rate-limiting enzyme in polyamine biosynthesis pathway, is characteristic of tumor cells. Similarly, prostaglandins (PGs) appear to be important in the pathogenesis of cancer because these affect mitogenesis, cellular adhesion, immune surveillance and apoptosis. Cancers form much more PGs than the original tissue from which they have arisen. This study has revealed that pretreatment of mice with resveratrol at a dose of 2.5 mg/kg body weight for two weeks blocked the N-nitrosodiethylamine (NDEA)-induced cytosolic ODC levels in the liver and lungs. The blockage was pronounced in hepatic tissue compared to pulmonary tissue. Resveratrol feeding caused a significant reduction in microsomal cyclooxygenase (COX) activities in the liver and lungs, while the dosage of NDEA (200 mg/kg body weight) induced COX activity 24 h after its administration. In any case, resveratrol pretreatment turned out to effectively block the induction of COX activity in the lungs by NDEA.

Key Words: resveratrol, ornithine decarboxylase, cyclooxygenase, N-nitrosodiethylamine

The process of carcinogenesis is multifunctional, multiphasic and multigenic in nature. A complex series of molecular and cellular events, which lead to the development of cancer, can be modulated by diverse endogenous and environmental factors (1, 2). Therefore, intervention of any or many events in this complex process by naturally occurring or synthetic chemical compounds should lead to a decreased cancer incidence (3). These compounds (chemopreventers), found in a variety of foods including fruits and vegetables, possess antimutagenic and anticarcinogenic properties (4, 5) and serve as preventive agents against lifestyle-related diseases such as cancer, cardiovascular disorders and cataract or brain dysfunction (6). Among them is resveratrol (3,4',5-trihydroxy-trans-stilbene), which has been identified as a major active compound of stilbene phytoalexin (7). Resveratrol is present in abundance in grapes, red wine, peanuts and mulberries, and takes part in prevention of human cardiovascular disease, modulation of lipid metabolism, and antioxidation of low-density lipoproteins etc. In addition, resveratrol has been reported to have anti-inflammatory and anticancer properties in a two-stage mouse skin cancer model (8). Its ability to inhibit the growth of several cell lines strongly suggests that the compound may also exert an inhibitory effect on cancer promotion/progression within the body (9–11).

Ornithine decarboxylase (ODC), an enzyme for polyamine biosynthesis, is important for tumor biology. ODC is at high level in cancer cells (12). Polyamines are implicated in diverse physiological processes that share a close relationship in cell proliferation and growth. In this connection, various studies using transgenic mice indicate that alteration in cellular polyamine levels modulates normal or cancer cell growth. Meanwhile, substantial evidence indicates a causal relationship between cancer development and overexpression of eicosanoid-forming enzymes (i.e., cyclooxygenases and lipoxygenases) in a variety of human and animal tumors (13). Inhibitors of cyclooxygenase have been found to protect colonic, mammary, esophageal, pulmonary and oral organs against tumorigenesis (14–16). Moreover, it is understood that genotoxic byproducts of cyclooxygenase (COX)-catalysed arachidonic acid metabolism (e.g. reactive oxygen species) contribute to ‘genetic instability’ and its subsequent malignant progression of malignant tumors (13). It thus seems likely that biosynthesis of polyamines and eicosanoids are made targets of therapeutic intervention. In the present study, we assessed the effects of resveratrol on the activity induction of ODC and COX by a potent chemical carcinogen “N-nitrosodiethylamine” in a mouse liver and lungs.

Materials and Methods

Material. N-nitrosodiethylamine (NDEA), dithiothreitol, pyridoxal 5-phosphate, DL-ornithine, resveratrol, 2,5-diphenyloxazole, 1,4-bis[5-phenyloxazole-2-oxazoyl]-benzene, 2,2'-p-phenylene-bis[5-phenyloxazole],
prostaglandins E₂ (PGE₂), D₂ (PGD₂) and F₂ₐ (PGF₂ₐ) were procured from Sigma Chemical Company, St. Louis, MO USA. DL-[1-14C] ornithine (specific activity, 56 mCi/mmol) and [1-14C] arachidonic acid (specific activity, 55 μCi/mmol) were procured from Amersham, U.K. F-254 TLC plates were purchased from Merck, Germany. All other reagents were of analytical grade and used without further purification.

Animals. Four to five week-old male Balb/c mice were procured from the Central Animal House of Post-graduate Institute of Medical Education and Research, Chandigarh, India. The animals were kept in the departmental animal house with a 12 h light/dark cycle and allowed free access to a pellet diet and drinking water.

Experimental protocol. The experimental design was approved by the Animal Experiment Committee of Post-graduate Institute of Medical Education and Research, Chandigarh. Mice were divided into the following four groups: ‘control’, ‘resveratrol-treated’, ‘NDEA-treated’ and ‘NDEA plus resveratrol-treated’ groups. In the in vivo experiment, resveratrol treatment was given orally at a dose of 2.5 mg/kg BW per day for 14 d with a solution of 0.625 mg/mL in 10% ethanol. NDEA was administered orally at a dose of 80 mg and 200 mg/kg BW in normal saline on the last day of resveratrol administration. The selection of this dose of resveratrol was based on the previous observation for its most effective method in inducing the antioxidant defense system (unpublished data). The two halves of control and NDEA-treated groups received their respective vehicles for resveratrol or NDEA. The mice were sacrificed 24 h after NDEA administration. Lungs and livers were removed after in situ perfusion. Tissues were homogenized in a teflon homogenizer at 4°C, with 3 volumes of 100 mM Tris-HCl buffer of pH 7.4 containing 5 mM dithiothreitol, 0.125 mM EDTA and 0.04 mM pyridoxal 5-phosphate for ODC assay and 0.05 M potassium phosphate buffer for PG assay and 0.05 M potassium phosphate buffer of pH 7.4 containing 10 mM EDTA and 1% BSA for COX assay, respectively. Homogenates were filtered through a cotton gauge, followed by centrifugation at 20,000×g for 10 min to obtain the cytosolic fraction in the former, and centrifugation of the 12,000×g supernatant at 105,000×g for 1 h at 4°C to obtain the microsomal pellet in the latter. The resulting microsomal pellet was washed three times with phosphate buffer to eliminate impurities such as BSA and EDTA, if any, prior to COX measurement (17).

ODC assay. The ODC activity was assayed by the method of Rao et al (18). Briefly, the reaction mixture containing 5 mM dithiothreitol, 0.4 mM pyridoxal 5-phosphate, 0.2 mM dl-ornithine (0.125 μCi dl-[1-14C]- ornithine), 1.5 mg of liver cytosolic protein or 0.5 mg of lung cytosolic protein in 0.250 mL of 0.1 M Tris-HCl buffer of pH 7.4 was incubated at 37°C for 60 min. Blanks contained heat-inactivated samples instead of enzyme sources. Reaction was stopped by the addition of 0.2 mL of 2 N H₂SO₄. Carbon dioxide liberated was trapped in a KOH solution. Radioactivity of 14CO₂ was measured in a toluene based scintillation fluid. ODC activity was expressed as pmol of CO₂ released/mg protein/h under the routine assay conditions.

In vitro studies. Animals treated with 80 mg or 200 mg NDEA/kg BW were sacrificed under light anesthesia in 24 h after carcinogen or vehicle administration. Lung and liver were perfused in situ and excised, from which both cytosolic fractions were subjected to ODC assay in the absence or presence of resveratrol at various concentrations in the same manner as mentioned above.

Cyclooxygenase assay. Cyclooxygenase activities were estimated using the lung or liver microsome according to a modified method of Lysz et al (19). Both lung (0.1 mg) and liver microsomes (1 mg) were used for the assay in reaction mixture containing 300 μL of 0.05 M potassium phosphate buffer containing 1 mM epinephrine, 1 mM glutathione (GSH) and 6 μM [1-14C] arachidonic acid (55 μCi/mmol). The reaction mixture was incubated at 37°C for 15 min. The reaction was terminated by adding 50 μL of 0.2 M HCl. Prostaglandins (PGs) were extracted three times with 0.5 mL ethyl acetate. Combined organic extracts were evaporated under nitrogen gas and the residue was redissolved in a minimum volume of ethyl acetate, which was applied to silica gel-60 F254 plates preactivated at 110°C for 1 h. TLC plates were developed with a chloroform/methanol/acetic acid/water (113 : 10 : 1.25 : 1, V/V/V/V) solvent. Heat-inactivated microsomes were run as blank to assess the nonenzymatic degradation of arachidonic acid, if any. Prostaglandin standards like PGE₂, PGD₂ and PGF₂ₐ were co-chromatographed with samples and were visualized by iodine vapor staining. The plates were cut and subjected to radioactivity measurement. The individual areas corresponding to standard PGs were separated and measured in precounted liquid scintillation vials containing 10 mL of scintillation fluid. The background value was subtracted from the count of each sample to obtain a true indication of biosynthesis. Formation of PGs was expressed as ng/mg protein/15 min.

Statistical analysis. Data were obtained as the means±SD, which were analyzed by one-way ANOVA and further by Scheffe's post-hoc test. Difference was considered significant at p<0.05.

Results and Discussion

Oral administration of NDEA to mice at a dose of 200 mg/kg BW caused significant induction of hepatic as well as pulmonary ODC (Fig. 1A). The degree of pulmonary ODC induction was 2.4 fold relative to 9.6 fold in that of hepatic ODC. The coexistence of resveratrol at 1 mM level in an ODC assay system, however, brought about a considerable decrease in hepatic ODC and inhibitory rate being two thirds of the net increment for hepatic ODC and in
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Fig. 1. In vitro inhibition of hepatic or pulmonary ODC activity from NDEA-treated mice by resveratrol (A) and its concentration dependence (B). Livers and lungs were excised immediately after in situ perfusion from mice dosed with 200 mg NDEA/kg BW on the preceding day, of which the cytosol fractions were prepared as enzyme sources for measurement of ODC activity. The enzyme reaction was carried out in the routine assay medium containing resveratrol at either 1 mM (A) or various concentrations (B). Values were obtained as the means ± SD for 3 or 4 assays; there is a significant difference between the presence and absence of resveratrol at * p < 0.001.

The present study shows that administration of NDEA to mice induced ODC activity differentially in the two organs. A single dose of NDEA at 80 mg/kg BW caused a slight induction in hepatic ODC in 24 h after its administration. However, other workers could notice the induction only by its dose at 200 mg/kg BW (20). In our study, a dose of 200 mg NDEA/kg BW was required for the induction of pulmonary ODC. The ODC induction was higher in the liver that occupies a main position in detoxication of xenobiotic compounds. It is known that the ODC induction is due to prolonged activation of cyclic AMP-dependent protein kinase, which leads to protein phosphorylation and ultimately leads to upregulation of transcription of ODC gene (21). Overexpression of ODC leads to increased production of polyamines which compensate for apoptosis due to their growth stimulatory effects and thus drive the cells in the proliferative pathway. Sometimes, in spite of ODC induction, a decrease in cellular polyamine level occurs as observed in dexamethasone-induced apoptosis (22). This decrease in polyamine arises from oxidation of spermidine and spermine by serum amine oxidase or intracellular EAD-dependent polyamine oxidase (23). In addition, H2O2 and aminoaldehydes produced during the catabolism of polyamines serve as strong inducers of apoptosis (24, 25). Since heterogeneous isoforms of ODC appear in tumorigenic organs, it is highly probable that NDEA causes upregulation/overexpression of tumor-specific ODC. Pronounced inhibition of hepatic ODC in vitro and in vivo by resveratrol may be accounted for by obstruction of such an ODC expression.

When COX activity was evaluated as the amounts of metabolites formed/mg protein/15 min. NDEA administration at a dose of 200 mg/kg BW did not show any effect on the total PGs formed by hepatic microsome, whereas the formation of total PGs by pulmonary microsome was significantly enhanced (p < 0.001) (Table 1). In contrast to this, treatment with resveratrol (2.5 mg/kg BW/d for 2 wk) significantly decreased individual (PGF2α, PGD2 and PGE2) and total PG formation by hepatic and pulmonary microsomes. NDEA alone did not affect the total PG formation by hepatic microsome, thereby those in control, resveratrol, NDEA and

Fig. 2. Effects of previous resveratrol feeding for 2 wk on NDEA-caused ODC induction. Male Balb/c mice of 4 or 5 wk age were fed a stock diet for 2 wk; in the meanwhile, resveratrol (0.625 mg/mL in 10% ethanol) was orally administered to half of the whole at a dose of 2.5 mg/kg BW/d and its vehicle to the remainder. At the 2nd wk, these two groups were further divided into equal parts. The mice of one part were individually treated with a single dose of 200 mg NDEA/kg BW, whereas the others were treated with saline alone. On the next day, all the mice were subjected to in situ perfusion. Immediately livers and lungs were excised and stored in deep freeze. When necessary, thawed tissues were homogenized in the usual way and separated into cytosolic or microsomal fractions by differential centrifugation. The cytosol fractions were used for ODC assay. Values were the means ± SD (n = 8); significantly different p < 0.001 * from the control without both NDEA and resveratrol, and # from the resveratrol-unfed NDEA group.

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Table 1. Effects of resveratrol feeding and/or N-nitrosodiethlymaine treatment of mice on enzymatic formation of prostaglandins from $[14C]$ arachidonic acid by the use of hepatic or pulmonary microsomes. The conditions of resveratrol feeding and/or NDEA administration and cell fractionation were the same as in Fig. 2. The microsomal fractions were used for COX assay. Hepatic and pulmonary COX activities were assessed as the amounts of prostaglandins formed under the routine assay procedure.

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<thead>
<tr>
<th></th>
<th>PGE$_2$</th>
<th>PGD$_2$</th>
<th>PGF$_{2\alpha}$</th>
<th>Total</th>
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</thead>
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<tr>
<td><strong>Hepatic COX activity</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>8.2±0.7</td>
<td>4.0±0.3</td>
<td>4.5±0.7</td>
<td>16.7±1.3</td>
</tr>
<tr>
<td>NDEA</td>
<td>9.4±1.0</td>
<td>3.8±0.3</td>
<td>4.3±0.6</td>
<td>17.5±1.8</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>4.2±0.7*</td>
<td>1.8±0.2*</td>
<td>2.1±0.1*</td>
<td>8.1±0.7*</td>
</tr>
<tr>
<td>Resveratrol+NDEA</td>
<td>5.9±0.5*#</td>
<td>3.5±0.3*</td>
<td>3.6±0.4*#</td>
<td>13.0±1.1*#</td>
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|                  |               |              |                 |               |
| **Pulmonary COX activity** |           |              |                 |               |
| Control          | 63.0±10.4     | 32.0±3.4     | 33.6±4.7        | 128±12        |
| NDEA             | 96.1±10.5*    | 40.9±3.5*    | 45.7±4.3*       | 183±15*       |
| Resveratrol      | 30.6±5.2*     | 16.5±2.5*    | 18.8±2.4*       | 66.1±6.6*     |
| Resveratrol+NDEA | 65.1±7.2*     | 31.0±4.0*#   | 41.0±4.9        | 137±15*       |

Values are the means±SD (n=5–6), significantly different from control (*), and NDEA (#) at p<0.05.

resveratrol+NDEA groups being obtained as 16.7±1.30, 8.05±0.76, 17.5±1.85 and 13.0±1.13 pmol/mg protein/15 min, respectively. Interestingly, the total PG formation by pulmonary microsome was significantly enhanced (p<0.001) in NDEA-administered mice. Nevertheless there was no significant increase in individual or total PG level in the NDEA+resveratrol group. The total PGs-forming capacities of pulmonary microsomes in control, resveratrol, NDEA and resveratrol+NDEA groups were 128±12, 66.1±6.58, 183±15.0 and 137±14.7 pmol/mg/15 min, respectively.

Metabolic pathway of COX-catalysed conversion of arachidonic acid to prostaglandins has been found to play an important role in every stage of carcinogenesis starting with activation of carcinogenic or genotoxic agents, tumor promoting effect, stimulation of metastasis, and weakening of immune response (26). COX-1 is expressed constitutively in most tissues, while COX-2, normally not detectable, is induced by various endogenous or exogenous agents and therefore referred to as an inducible form (27). A few cases are known where COX-2 constitutively occurs in certain cells (28). For this reason, it was of interest to examine whether COX would actually be induced by NDEA or modulated by resveratrol. It has been previously shown by Jang et al. (8) that resveratrol inhibits both COX-1 activity and its related hydroperoxidase function in seminal vesicle microsomes, thereby exerting antitumorigenicity. In the present in vivo study, we have found that NDEA induces pulmonary COX but not hepatic COX. Resveratrol treatment led to a marked reduction in COX activity corresponding to approximately 50% of the control, irrespective of lung or liver, and pulmonary PG levels remained unaltered by NDEA administration to resveratrol-pre-treated mice. The extent of COX modulation by resveratrol indicates that its activity is mainly attributable to COX-1.

Though COX-1 was speculated to be a housekeeping enzyme, there were no gastric and renal pathologies in COX-1 deleted mice. Its deficiency affected only parturition, while COX-2 deleted female mice were essentially infertile concurrently with cumulus stigmata formation and abnormal ovulation (27). Renal abnormality was observed in all adult COX-2 deleted mice (29–32) and the renal pathology became more severe with age, resulting in the last stage of renal disease (27). Therefore, the designation “COXs” as expressions of housekeeping and/or response genes may not be entirely accurate. Actually COX-1 as well as COX-2 can contribute to PG formation during an inflammatory response, and their contributory proportions may depend upon the inflammatory stimuli, the time of insult, and the existence ratio of isoforms in a target tissue. COX-2 derived PGs appear to have important roles not only in the resolution or healing phase but also in the early stage of inflammatory process. Thus, resveratrol preferentially inhibiting inherent COX-1 may well serve as a chemopreventive against cancer at various stages. This inference is supported by the finding that both COX deficiencies decreased the number of intestinal polyps by 80% in a mouse model of spontaneous intestinal adenomas (33).

In conclusion, we first observed the in vivo inhibitory effects of resveratrol on NDEA-induced ODC and PG biosynthesis. The inhibition of these ODC and COX activities would likely be responsible for the in vivo anticiarcinogenic effect of resveratrol.

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REFERENCES


