Anticancer Effect of Rosiglitazone, a PPAR-γ Agonist against Diethylnitrosamine-Induced Lung Carcinogenesis

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ABSTRACT: Multiple effects on cancer cells are exerted by the peroxisome proliferator-activated receptor γ (PPAR-γ). Recent studies have shown that rosiglitazone, a synthetic PPAR-γ ligand, inhibits the growth of cells. This research was designed to assess the impact of rosiglitazone on diethylnitrosamine (DENA)-induced lung carcinogenesis in Wistar rats and to study the underlying molecular mechanism. A total of 40 adult male Wistar rats were separated into four groups as follows: group 1 is known as a control. Group 2 is known as the DENA group (150 mg/kg, i.p.). Group 3 and group 4 denote DENA-induced rats treated with 5 and 10 mg/kg rosiglitazone, respectively. Lipid peroxidation, various antioxidant enzymes, histological perceptions, and caspase-3, Bcl2, and Bax gene expression were measured in lung tissues. Rosiglitazone treatment reverted the DENA-induced changes in the expression of these genes, inflammatory cytokines, and oxidative stress. However, blotting analysis discovered reduced caspase-3 and BAX expressions and elevated Bcl-2 expression in DENA-induced rats. The expression of such proteins causing DENA lung cancer was restored by rosiglitazone therapy.

INTRODUCTION

Lung cancer is the world’s leading cause of cancer death. Although numerous diagnosis and treatment strategies have been developed for lung cancer, the overall five-year survival has not increased considerably because of poor forecasts and lack of effective methods for early detection. New treatment strategies for lung cancer, especially molecular therapies, and the survival rate for patients with lung cancer must be increased urgently. In addition, increased knowledge of essential molecular modifications in normal cells leading to unstable and malignant tumor cells may contribute to the development of possible treatments for this disease. Lung cancer’s major risk factors include air, aflatoxins, food additives, water, industrial toxic chemicals, alcohol, and environmental pollutants. Diethylnitrosamine (DENA) is known to be a lung cancer agent in smoke, cheddar cheese, cured meal, drinking water, fried foods, pesticides, and cosmetics in the field of agriculture and pharmaceuticals.1,2 DENA causes lung cancer in laboratory animal models by inhibiting several enzymes involved in the DNA repair process. In rats, DENA is a powerful pulmonary carcinogen that affects the initiation of carcinogenesis during an enhanced cell proliferation cycle with pulmonary necrosis. DENA-mediated free radical production, increased lipid peroxidation (LPO), endogenous antioxidant depletion, cytotoxicity, and carcinogenesis are recorded in several studies.3,4

The nuclear hormone receptor peroxisome-activated receptor γ (PPAR-γ) provides a strong link between lipid metabolism and gene transcription regulation. A new class of antidiabetical medication is now widely prescribed, and a group of PPAR-γ activators are now commonly prescribed to control growth arrest and terminal differentiation of adipocytes. Several ligands, such as rosiglitazone, pioglitazone, troglitazone, and 15-deoxide-presaturated J2, have been identified, and some polyunsaturated fatty acids are known. In several organ groups, PPAR-α is expressed: intestines, adipose, pulmonary tissue, breasts, and liver. Several studies have shown that PPAR-β ligand cancer cells can induce cell differentiation and apoptosis and have proposed potential uses as chemopreventive carcinogenesis agents.5,6 This together led us to start these research studies to gain insights into the
Possibility of rosiglitazone supplement safety based on the mechanism against DENA-induced lung cancer.

**RESULTS AND DISCUSSION**

**Effect of Rosiglitazone on LPO and Antioxidant Enzymes.** Determining thiobarbituric acid reactive substances was used to determine LPO in the fresh lung homogenate. In the group DENA, the amount of malondialdehyde (MDA) (an LPO marker) increased 341.1% but was lower at 27.4 and 71.8% following the 5 and 10 mg/kg supplementation of rosiglitazone, respectively (Figure 1A). In the DENA group, reduced glutathione (GSH) levels (an antioxidant marker) decreased by 71.3% but increased by 76.9 and 193.8% after 5 and 10 mg/kg supplementation of rosiglitazone, respectively. The activity of another antioxidant marker, Gpx, decreased in the DENA group by 63.2%. However, after 5 and 10 mg/kg of rosiglitazone supplementation, the activities of Gpx increased by 69.3 and 184.5%, respectively. The activity of the additional antioxidant marker superoxide dismutase (SOD) in the DENA group was reduced by 61.3% and increased by 27.4% and 104.2%, respectively, after 5 and 10 mg/kg of rosiglitazone supplementation. Catalase (CAT) activity, the marker for antioxidants, decreased in DENA groups by 73.5% but increased by 84.3 and 193.2%, respectively, as a consequence of 5 and 10 mg/kg rosiglitazone supplementation (Figure 1B).

**Effect of Rosiglitazone on Inflammatory Cytokines.** In the DENA group, the TNF-α rate was increased by 284.7% but reduced by 31.6 and 106.3% after 5 and 10 mg/kg of rosiglitazone supplementation, respectively (Figure 2A). The DENA group showed an increase in IL-1β by 327.8%, but reduced by 28.4 and 94.8% following 5 and 10 mg/kg of rosiglitazone supplementation (Figure 2B). Like IL-6, IL-1β and TNF-α levels were also amplified by 197.8% in the DENA group.
group but decreased, respectively, by 27.6% and 74.0% from 5 to 10 mg/kg rosiglitazone supplementation (Figure 2C).

Cytokines have important roles in host defense and pathophysiology under inflammatory conditions. After administration of DENA in rats, the development of IL-6, IL-1β, and TNF-α has increased suggestively in this investigation.13−15 Western blot analysis has shown that the administration of DENA significantly increased pro-apoptotic Bax protein expression and decreased Bcl-2 expression. Cytochrome c has been released into the mitochondrial cytosol, and then, caspase-3 expression was increased.16,17 This causes apoptosis of the tumor cells. Both effects can enhance the chemotherapy effect and lower the dose-dependent toxicity of DENA in combination with rosiglitazone (5 and 10 mg/kg).

**Effect of Rosiglitazone on mRNA Expression of Caspase-3, Bax, and Bcl-2.** The downstream caspase function in both the nucleus and the targets for the cytosol is caspase-3, a central executor of apoptosis in programmed cell death. To test the hypothesis of a lower level of Caspase-3 in rat because of rosiglitazone, quantitative real-time polymerase chain reaction (qRT-PCR) and western blotting analysis were performed on the mRNA and Caspase-3 protein expressions. In comparison with the control group, as shown in Figure 3A−C, the caspase-3 mRNA and protein levels were considerably higher in the DENA-driven rats, while the dose-dependent treatment was substantially reversed by rosiglitazone (5 and 10 mg/kg). Accumulating studies have shown that the increase of proapoptotic protein Bax and decrease of antiapoptotic protein Bcl-2 promoted cytochrome c release in mitochondria, and therefore activated the cascades of apoptosis.

Western blot analysis demonstrated that DENA administration substantially increased the expression of the proapoptotic protein Bax and diminished the expression of the anti-apoptotic protein Bcl-2. Cytochrome c has been released into the cytosol from the mitochondria, which increases caspase-3 protein expression.16,17 It induces apoptosis of the tumor cells. These effects can improve the chemotherapy effect and lower the dose-dependent toxicity of DENA in combination with rosiglitazone (5 and 10 mg/kg).

**Effects of Rosiglitazone on DENA-Mediated Lung Histopathological Changes.** The lungs were isolated at 24 h after administration of rosiglitazone in the lung tissue to assess histological changes following rosiglitazone post treatment in DENA-challenged rats. The control group’s lung tissues had a normal structure, and there were no histopathological changes. Histological examination of the DENA group by hematoxylin and eosin (H&E) staining revealed serious pulmonary oedema, stroma hemorrhagia, alveolar collapse, and mass inflammatory cell infiltrations, which were seriously destructive of the lung. Nonetheless, after treatment with rosiglitazone (5 and 10 mg/kg), effective alleviation of lung structure degradation was observed, depending on the dosage (Figure 4).

#### CONCLUSIONS

In brief, the results of this study showed that rosiglitazone can reduce lung carcinogenesis induced by DENA by down-regulating LPO, inflammatory cytokines such as IL-6, IL-1β, and TNF-α, and pro-apoptotic factors Bax, whereas upregulating antioxidant enzyme levels such as SOD, CAT, Gpx, GST, and GSH and the anti-apoptotic factor Bcl-2. Further clinical study is required to find out an exact effect.

#### MATERIALS AND METHODS

**Chemicals.** DENA and rosiglitazone have been acquired from Sigma-Aldrich. The cell signaling technique was used to acquire both primary and HRP-conjugated secondary antibodies. The western blotting kit has been obtained from Abcam, USA. All other chemicals used were of analytical quality.
Experimental Animals. This study was conducted on male Wistar rats (220 ± 10 g). All the animals were procured from and maintained in the central animal house of People’s Hospital of Ningjin County, China. Animals were caged in groups with the normal 12 light/dark cycle maintained at 24 ± 2 °C temperature. The animals were served pelleted rat chow and water ad libitum, available commercially. All animal procedures were approved by the animal ethical committee of People’s Hospital of Ningjin County (AECPN NO: AECP/11827/2019). The experiment was carried out according to the guidelines of the NIH at the People’s Hospital of Ningjin County.

Experimental Design. As described earlier, the DENA-induced animal model of lung cancer has been developed. The animals were intraperitoneally (i.p.) given 150 mg/kg body weight dosage of DENA for 21 days once in 7 days. The rats have been split into four different categories, comprising 10 rodents per group, randomly following the induction of lung cancer: Group 1 was treated as a normal control and only distilled water not exceeding 1 mL was given orally. Group 2 was i.p. given 150 mg/kg DENA. In groups 3 and 4, rats were treated with DENA orally for 15 days with 5 and 10 mg/kg rosiglitazone, respectively. Feed was deprived overnight for 24 h after the last operation, and all rats were anesthetized. Jugular vein blood was obtained and serum was isolated and used for the biochemical investigation. For histopathological analysis, H&E stained sections were examined with the light microscope at a magnification of 100×.

Measurement of LPO and Antioxidant Enzymes. LPO was measured in fresh pulmonary homogenates according to Quintero-Garcia et al. by the detection of thiobarbituric acid reactants. The final product of LPO has been determined by measuring the absorption at 534 nm. A measurement of the absorption of CAT activity at 420 nm was performed. The specimens were supplied with 500 μL of phosphate buffer, serum, and liquid. The absorption was estimated at 560 nm for SOD. A specimen with phosphate (1.2 mL), homogeneous tissue (0.1 mL), nitroblue tetrazolium (0.3 mL), and NADH (0.2 mL) was used. Following the procedure of the GSH content was determined by the Ellman reaction in the lung tissue homogenates. At 412 nm, the final product was measured. The absorption was measured at 340 nm to determine the activity of Gpx in the tissue homogenate.

Measurement of Inflammatory Cytokines. IL-6, IL-1β, and TNF-α levels in serum were measured using rat cytokine (Xitang Biotechnology Co., Ltd., Shanghai, China) kits, which are commercially available immunoassay systems [enzyme-linked immunosorbent assay (ELISA)]. The experiments with ELISA were performed following strict directions.

Quantitative Real-Time Polymerase Chain Reaction. A total DNA protein kit (E.Z.N.A.) was utilized for total lung RNA extraction. A BCA protein assay kit has been used to assess protein concentrations. Total RNA (1 μg) was reverse-transcribed with an ImProm-II reverse transcription system package. For mRNA amplification of apoptosis-related genes using the following front and reverse primers (Table 1), an ABI PRISM 7500 sequence detection system was applied. The conditions for amplification are 30 s at 95 °C and then 39 cycles of 5 s at 95 °C, 30 s at 58 °C, and 34 s at 72 °C. Caspase-3, Bax, and Bcl-2 levels of mRNA are standardized to β-actin levels. Triplicate studies have been performed. All data were examined with the 2−ΔΔCt process (ΔCt = CtTarget gene − Ctβ-actin, ΔΔCt = ΔCtexp − ΔCtControl).

Histopathological Study. The method by Fukushima et al. has been used in histopathological study of the lung tissue. The lower lobe of the lung was soaked in 10% formalin and immersed in paraffin. Tissues are cut to 3 μm thickness and treated with H&E. A tissue section under a light microscope was then examined. Sections are tested under a light microscope at a magnification of 100×.

Western Blotting. Equal amounts of the total protein are filled in 80 V sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels for 80 min, electrically transferred to polyvinylidene fluoride membranes by the wet transfer method (250 mA, 90 min), and blocked in 5% bovine serum albumin at 4 °C overnight. Subsequently, the membranes were incubated with an anti-β-actin antibody (dilution 1:1000), anticaspase3 antibody (dilution 1:200), anti-bcl-2 antibody (dilution 1:100), and anti-bax antibody (dilution 1:500) at room temperature for 2 h. After TBST washing, membranes were incubated for 1 h at room temperature with secondary goat anti-mouse IgG (dilution 1:1000) and then combined with horseradish peroxidase. Equal protein loads were verified with anti-β-actin antibody on each lane. The reagent chemiluminescence was then observed with proteins. Bio-Rad Quantity One v4.62 was used to calculate the density of

| name                 | sequence (5′ → 3′)                                      |
|----------------------|-------------------------------------------------------|
| Caspase-3            | forward primer: CGGAGCTTGGGACGCAGAG                  |
|                      | reverse primer: ACACAAGCCCATTTGCAGGTT                |
| Bax                  | forward primer: ACACACGCAGACAGACAGCC                 |
|                      | reverse primer: GTGTAACGCAGCGAAGGG                    |
| Bcl-2                | forward primer: GAATTCCTCTCCCACATCGGCC              |
|                      | reverse primer: TGTTTTCTTTTGCGCGCGGT                |
| β-actin              | forward primer: CCGACCATGTAAGACTAAGCA               |
|                      | reverse primer: CCGCTCCGAGTGCCATCAC                 |

Table 1. Primer Sequence for RT-PCR

Figure 4. Histopathological images of the effect of rosiglitazone on DENA-induced carcinogenesis in lung tissues of Wistar rats (H&E; 200×).
the protein band. Protein expression levels were normalized internally with β-actin.

**Statistical Analysis.** All test data are shown as standard deviation (SD) and means. Experimental results are analyzed and compared by means of analysis of variance (ANOVA), followed by the post hoc Tukey test, which showed P < 0.05, suggesting statistical significance for group mean comparisons. SPSS for Windows, version 22, has been used for all statistical analysis.

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**Notes**

The authors declare no competing financial interest.

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### ABBREVIATIONS

Bax, Bcl2-associated X protein; Bcl2, B-cell lymphoma 2; CAT, catalase; DENA, diethylnitrosamine; ELISA, enzyme-linked immunosorbent assay; Gpx, glutathione peroxidase; IL-1β, interleukin 1 beta; IL-6, interleukin-6; GSH, reduced glutathione; MDA, malondialdehyde; PPAR-γ, peroxisome proliferator-activated receptor γ; qRT-PCR, quantitative real-time polymerase chain reaction; ROS, reactive oxygen species; SD, standard deviation; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-alpha

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