

Analysis of the effect of rutin on GSK-3 β and TNF- α expression in lung cancer

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Abstract. The aim of the present study was to investigate the effect of rutin treatment on the expression of glycogen synthase kinase (GSK)-3 β and tumor necrosis factor (TNF)- α in A549 human lung carcinoma cells. The A549 cells were divided into control, cisplatin and rutin (low, middle and high) groups. ELISA and western blot analysis of TNF- α expression, 4',6-diamino-2-phenylindole (DAPI) staining and GSK-3 β immunofluorescence staining were used to investigate the effect of rutin in the human lung carcinoma cells, using cisplatin as a positive control. TNF- α expression was significantly higher in the rutin and cisplatin groups compared with the control group. Additionally, DAPI staining revealed that the number of apoptotic cells was higher in the rutin and cisplatin groups compared with the control group, and immunofluorescence showed that the expression of GSK-3 β in the cisplatin and rutin groups was significantly higher compared with that in the control group. The results of the present study suggest that rutin promotes the TNF- α -induced apoptosis of A549 human lung carcinoma cells. Furthermore, rutin may be able to regulate the expression of GSK-3 β protein in these cells.

Introduction

Lung cancer is a disease with high morbidity and mortality rates (1). The prevalence of lung cancer is increasing in

China, particularly in large cities (2). Data released by the World Health Organization in 2003 indicated that lung cancer is one of the most malignant cancers, and seriously affects the health and mortality of patients (3). Tumor necrosis factor (TNF)- α is indicated to be a key cytokine for use in the treatment of cancer (4). TNF- α has been found to have direct antitumor effects and strong biological activity (5). Furthermore, elevated levels of TNF- α have been found to be associated with the development and treatment of lung cancer (6). Glycogen synthase kinase (GSK)-3 β has been suggested as a therapeutic target for numerous diseases, including cancer, because of its diverse cellular functions (7). GSK-3 β phosphorylates a variety of proteins associated with cell cycle regulation, apoptosis and cell survival (8); therefore, the regulation of GSK-3 β expression may have an important role in the prevention and treatment of lung cancer.

Rutin is a pharmacological agent that has been used clinically to regulate cardiovascular disease for many years and may be effective in the treatment of tumors (9,10). In the present study, the effect of rutin treatment on GSK-3 β and TNF- α expression in lung cancer was investigated.

Materials and methods

Materials. 4',6-Diamino-2-phenylindole (DAPI) and antibodies against TNF- α (MK1169) and GSK-3 β (27C10) were purchased from Wuhan Boster Biological Technology, Ltd. (Wuhan, China). Other reagents were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The MCO-5AC CO₂ thermostat incubator was obtained from Sanyo Electrical Biomedical Co., Ltd. (Osaka, Japan).

Cell culture. A549 lung carcinoma cells were provided by the School of Pharmaceutical Sciences of Jilin University (Changchun, China). The A549 cells were maintained in plastic dishes (150 mm) with RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Shanghai BaiJin Chemical Group Co., Ltd., Shanghai, China), 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. The cells were divided into five groups: Control, cisplatin and rutin (low, medium and high) groups. Cells were seeded into 96-well plates (5 \times 10⁻⁵ cells/well) in Dulbecco's modified Eagle's medium

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(Santa Cruz Biotechnology, Inc., Dallas, TX, USA) supplemented with 10% fetal bovine serum and incubated with 1×10^{-8} (low), 2×10^{-8} (medium) or 4×10^{-8} mol/l (high) rutin or 1×10^{-9} mol/l cisplatin (both from Sigma-Aldrich; Merck KGaA) for 24 h. Control cells were treated with PBS under the same culture conditions.

ELISA. Cells were lysed using tissue Total Protein Lysis Buffer (Yi Li Biotechnology Co., Ltd., Shanghai, China) and centrifuged at 10,000 to 14,000 \times g for 15 sec at 4°C. Lysates were subsequently analyzed according to the instructions of a TNF- α ELISA kit (EH3TNFA; Nanjing Zhi Bai Cui Biology Technology Co. Ltd., Nanjing, China), with measurement of the absorbance value at 450 nm.

Western blot analysis. A549 cells were homogenized in radioimmunoprecipitation assay buffer (Sigma-Aldrich; Merck KGaA). Cell suspensions were centrifuged at 10,000 to 14,000 \times g for 4°C at 15 sec. The total protein concentration of the homogenates was measured using a bicinchoninic acid assay reagent. Equal amounts of protein extract (20 μ l) were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes via electroblotting. Separated proteins were transferred to polyvinylidene difluoride membranes (Sigma-Aldrich; Merck KGaA). After blocking with 5% non-fat milk (4°C, 2 h), the membranes were probed with primary antibodies against TNF- α (1:500; MK1169; Wuhan Boster Biological Technology, Ltd.) and GAPDH (1:500; SAB2100894; Sigma-Aldrich; Merck KGaA) at 20–27°C for 2 h, followed by incubation with anti-mouse immunoglobulin G secondary antibodies (1:400; AP130P; Sigma-Aldrich; Merck KGaA) at 37°C for 20 min. Immunocomplexes were visualized using an enhanced chemiluminescence detection system (EMD Millipore, Billerica, MA, USA). Immunoreactive bands were analyzed using Image Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Immunofluorescence and DAPI staining. Cells were fixed with 4% paraformaldehyde (Sigma-Aldrich; Merck KGaA) for 15 min at room temperature, then washed three times with PBS for a total of 10 min. Cells were incubated with GSK-3 β antibody (1:200) at 4°C overnight. Following this, the cells were washed three times for 5 min and subsequently incubated with anti-mouse immunoglobulin G secondary antibodies (1:500; AP130P; Sigma-Aldrich; Merck KGaA) at 4°C for 2 h. DAPI was then used for nuclear staining. Cells were mounted and images were captured using a Nikon Eclipse 80i fluorescence microscope (Nikon Corporation, Tokyo, Japan). The protein expression of GSK-3 β and the number of apoptotic cells (DAPI) were analyzed using Image Pro Plus 6.0 software.

Statistical analysis. All data were obtained from at least three separate experiments and are presented as the mean \pm standard error of the mean. Statistical comparisons were made using the Student's t-test. Statistical analysis of the data was performed using SPSS version 11 (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Table I. TNF- α content in the five groups.

Groups	TNF- α (ng/l)
Control	50.49 \pm 2.02
Cisplatin	229.37 \pm 27.58 ^a
Rutin (low)	114.09 \pm 9.40 ^a
Rutin (medium)	160.03 \pm 18.55 ^a
Rutin (high)	154.73 \pm 10.00 ^a

^a $P < 0.01$ vs. control. TNF, tumor necrosis factor.

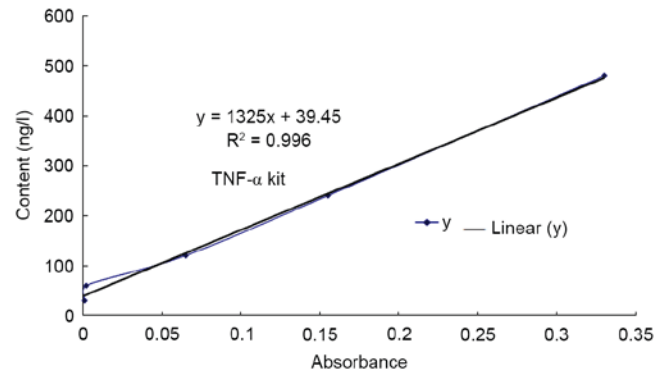


Figure 1. TNF- α standard curve in lung carcinoma A549 cells. TNF, tumor necrosis factor.

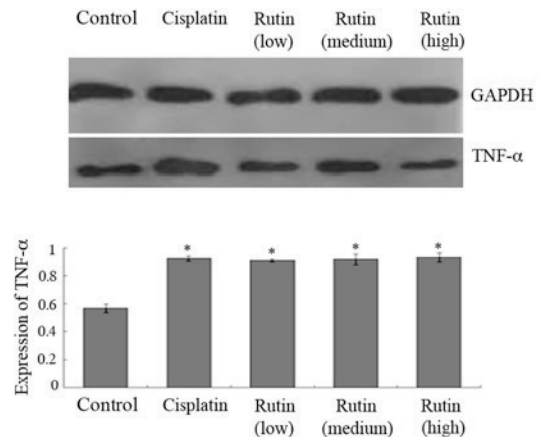


Figure 2. Analysis of the expression of TNF- α relative to the GAPDH reference protein using western blotting. ^a $P < 0.05$ vs. the control group. TNF, tumor necrosis factor.

Results

TNF- α content determined using ELISA. An ELISA kit was used to assess the expression of TNF- α in different groups. The results of the ELISA demonstrate that the expression of TNF- α protein in the cisplatin group was significantly increased compared with that in the control group ($P < 0.01$; Fig. 1, Table I). Furthermore, the expression of TNF- α in the low, medium and high rutin groups was also increased significantly compared with that in the control group ($P < 0.01$; Table I).

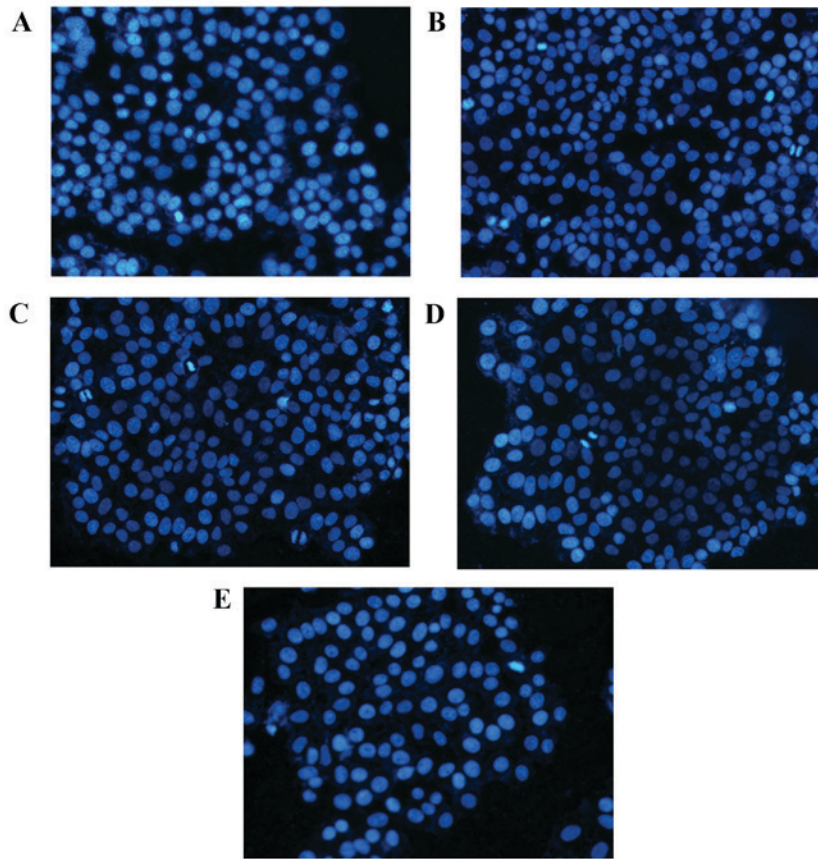


Figure 3. Immunofluorescence 4',6-diamino-2-phenylindole staining. (A) Control group, (B) cisplatin group, (C) low rutin group, (D) medium rutin group and (E) high rutin group. Magnification, x200.

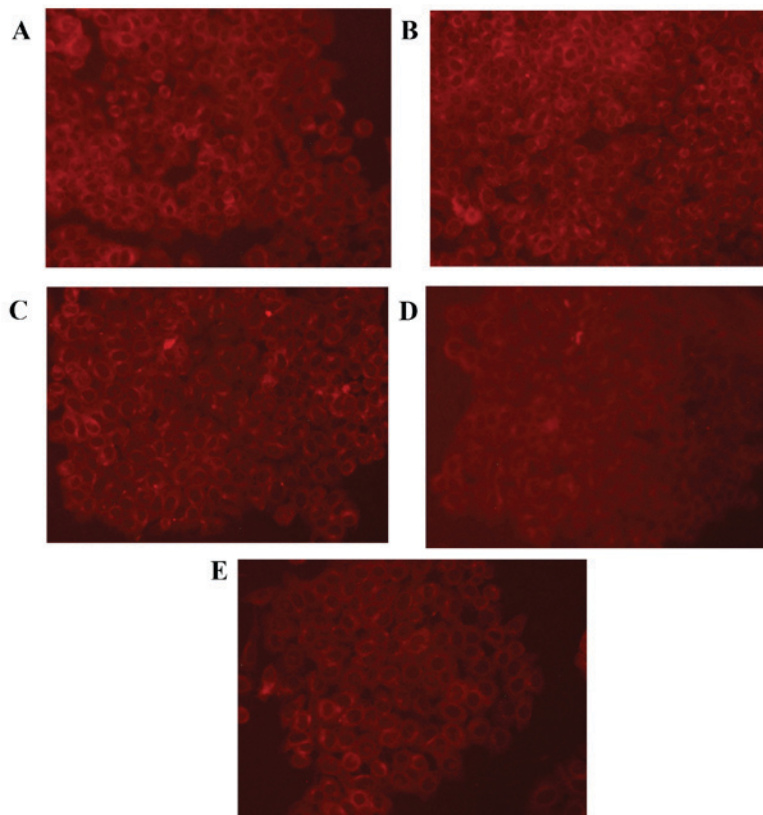


Figure 4. Glycogen synthase kinase-3 β expression detected by immunofluorescence staining (red). (A) Control group, (B) cisplatin group, (C) low rutin group, (D) medium rutin group and (E) high rutin group. Magnification, x200.

Western blot analysis. Western blotting was conducted to further investigate the expression of TNF- α . The results were analyzed semi-quantitatively according to the grayscale value. TNF- α levels were significantly increased in the cisplatin group compared with the control group (Fig. 2; $P < 0.05$). Additionally, the expression of TNF- α in the low, medium and high rutin groups was also significantly higher compared with that in the control group (Fig. 2; $P < 0.05$).

DAPI staining. DAPI staining results (Fig. 3) demonstrated that cells in the control group had uniform chromatin, a large nucleus and integrity of the nuclear envelope. By contrast, apoptotic morphological changes of A549 cells after 48 h rutin or cisplatin treatment were observed. Relative to the control group, cells groups treated with rutin and cisplatin exhibited higher numbers of detached cells with round and shrunken morphologies and condensed nuclei.

Expression of GSK-3 β protein. To validate the expression pattern of GSK-3 β in lung cancer cells, GSK-3 β specific fluorescent staining was performed on A549 cells (Fig. 4). The results of the staining assay revealed that the cells in the control group had uniform chromatin and a large nucleus. Relative to the control group, levels of GSK-3 β protein were markedly increased in the cisplatin group. In addition, GSK-3 β protein expression was increased in the low, medium and high rutin groups compared with the control group.

Discussion

Human TNF- α is composed of 233 amino acids (molecular weight, 26 kDa) and contains a signal peptide composed of 76 amino acid residues (11). It has previously been suggested to be an important tumor-related factor (12). One of the aims of the present study was to determine whether rutin promotes TNF- α expression. The results of the present study demonstrated that TNF- α expression was significantly increased in the low, medium and high rutin groups compared with the control group. These results suggest that rutin may stimulate the expression of TNF- α in A549 human lung carcinoma cells, and serve a role in killing tumor cells.

GSK-3 is a serine/threonine kinase that is highly evolutionarily conserved and is present in many mammalian eukaryotic cells, functioning to remove active glycogen synthase, which regulates cell differentiation, proliferation, survival and apoptosis (12). GSK-3 was first isolated from rabbit skeletal muscle tissues and there are two subtypes; GSK-3 α and GSK-3 β (13). GSK-3 β is a key enzyme associated with glycogen metabolism (14). This in turn affects mitochondrial permeability and the release of cytochrome *c*, which is associated with apoptosis regulation (13). In the present study, the expression of TNF- α was significantly increased in the rutin groups compared with the control group and the expression of GSK-3 β was increased concurrently, which indicates that TNF- α may have promoted GSK-3 β protein expression in the rutin group. These results suggest that rutin stimulated the expression of TNF- α .

In conclusion, the results of the present study suggest that rutin may serve as an effective antitumor treatment. Rutin treatment may increase the expression of TNF- α , and promote the expression of GSK-3 β .

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