Effects of Feiyanning Decoction, a compound traditional Chinese medicine, on iNOS and COX-2 expressions induced by tumor necrosis factor-α in lung adenocarcinoma cell line

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OBJECTIVE: To study the effect of Feiyanning Decoction (FYN), a compound traditional Chinese medicine, on expressions of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) activated by tumor necrosis factor-α (TNF-α) in human lung adenocarcinoma epithelial cell line (A549).

METHODS: A549 cells were incubated with rat serum containing FYN for 24 h. Gene expressions of iNOS and COX-2 were determined by quantitative real-time polymerase chain reaction and Western blot. The iNOS-dependent luciferase reporter was transfected for 24 h and the cells were treated with the reagents for 24 h, then the transcriptional activity of iNOS promoter was detected by luciferase assay. The production of NO was determined by diaminofluorescein-2.

RESULTS: FYN significantly inhibited TNF-α-induced expression of iNOS and COX-2 compared with the control group in A549 cells (P<0.01, P<0.01). Also, FYN inhibited the transcriptional activity of the iNOS promoter and reduced NO production compared with the control group (P<0.01, P<0.01).

CONCLUSION: These results suggest that FYN inhibits iNOS and COX-2 activation induced by TNF-α, therefore, it is expected to develop a new strategy to treat lung cancer.

KEYWORDS: Lung neoplasms; nitric oxide synthase; cyclooxygenase 2; tumor necrosis factor-alpha; adenocarcinoma; compounds; rats

Lung cancer is one of the most common cancer-related causes of death worldwide[1]. Natural products including those from plants and microorganisms provide rich potential for discovery of antitumor drug. Feiyanning Decoction (FYN) is a preparation containing water-soluble components of Huangqi (Radix Astragali Mongolici), Ganchan (Succisa Bafo), Fengfeng (Nidus Vespi), Shanyao

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(Rhizoma Polygonati Sibirici), Yinyanghuo (Herba Epimediit), etc. Both clinical and laboratory studies showed that FYN has anti-lung cancer effect by its anti-inflammatory activity[4,5]. However, there is limited information regarding the molecular mechanisms of FYN against lung cancer cells. The purpose of the present study was to examine the effects of FYN on expression of inducible nitric oxide synthase (iNOS) activated by tumor necrosis factor-α (TNF-α) in lung adenocarcinoma cell line (A549) by using diaminofluorescein-2 (DAF-2), real-time polymerase chain reaction (RT-PCR), Western blot and luciferase assay methods.

1 Materials and methods

1.1 Materials FYN (Huangqi 40 g, Ganqian 9 g, Fengfong 9 g, Yinyanghuo 15 g, etc) was prepared by the Pharmacy of Longhua Hospital, Shanghai University of Traditional Chinese Medicine based on previous report[4]. FYN was condensed with water bath to 2.0 g/mL per crude drug content and stored at 4 °C. Recombinant TNF-α was purchased from Calbiochem, Germany. Dimethyl sulfoxide (DMSO) was purchased from Santa Cruz, USA. DAF-2 was purchased from Daiichi Pure Chemicals Co., Ltd, Japan. Krebs-ringer phosphate (KRP) buffer consists of sodium chloride (120 mmol/L), potassium chloride (4.8 mmol/L), calcium chloride (0.54 mmol/L), magnesium sulfate (1.2 mmol/L), glucose (11 mmol/L), and pH 7.2 sodium phosphate (15.9 mmol/L). Fetal bovine serum (FBS) was purchased from Shanghai Huamei Biotechnology Company, China. Roswell Park Memorial Institute (RPMI) 1640 medium and 0.25% trypsin were purchased from Gibco Company, USA. Lipofectamine™ Plus Reagents kit was purchased from Invitrogen Corporation, USA. Dual-luciferase reporter gene assay system was purchased from Promega, USA. Lumat 9507 fluorescence detector was purchased from Beijing Xinfeng Electromechanical Technology Company, China.

1.2 Methods

1.2.1 Serum-containing medium preparation A total of 16 male Wistar rats were obtained from the Shanghai Slac Laboratory Animal Co., Ltd. with (300 ± 20) g body weight and the permit number was SCXK (Shanghai) 2007-0005. They were randomly divided into two groups with eight in each group. Rats in the control group were administered with equal volume of normal saline twice daily while rats in the treatment group were administered with FYN at a dose of 22 mL/kg daily (equivalent to eight times of the clinical dose in human body) based on body weight[4,5]. According to the serum preparation protocol[4,6], rats were administered with drugs daily by oral gavage for 3 d. The serum was inactivated for 30 min in a 56 °C water bath, then filter-sterilized through a 0.22 μm membrane filter. Serum was stored at −20 °C until use.

1.2.2 Cell culture The human lung cancer cell line A549 was originally obtained from the American Type Culture Collection and maintained in 5% CO2 at 37 °C in RPMI 1640 medium containing 10% FBS. The cells were divided into control group, TNF-α (1 μg/L) group, FYN alone group (15 μg serum containing FYN), and FYN plus TNF-α group.

1.2.3 RT-PCR Cell lysates were prepared by RNeasy Lysis Buffer (Qiagen Valencia, CA) from each experimental condition. It was first homogenized by passing them through a QIAshredder spin column (Qiagen, Valencia, CA). The total DNA and RNA fractions were further isolated using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. The isolated total RNA was reverse-transcribed into complementary DNA (cDNA) using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Gene expressions were analyzed by quantitative RT-PCR using the SYBR Green PCR Master Mixture with the ABI PRISM 7000 sequence detection system and relative quantification software (Applied Biosystems, Foster City, CA). β-actin was used as the housekeeping gene. The primers are listed in Table 1.
1.2.4 The DAF-2 assay  DAF is fluorescent NO indicators. The reaction of NO and DAF yields the corresponding bright green-fluorescent triazolofluoresceins. It is feasible to detect the formation of NO. Determination of NO was performed as described by Tokoro et al[41]. Briefly, the A549 cells were seeded onto 96-well flat-bottomed culture plates for 6 h to make the A549 adherent to the plates. The cells were cultured for 24 h in 200 µL fresh medium containing reagents at 37 °C. The adherent cells were washed twice with KRP buffer, and then DAF-2 (10 µmol/L), L-arginine (1 mmol/L), and N^6^-amino-L-Arginine L-NAME, 10 mmol/L) dissolved in 200 µL of KRP buffer were added. After incubation for further 2 h, the supernatants were transferred to black microplates and the fluorescence was measured with a fluorescence microplate reader calibrated for excitation at 485 nm and emission at 538 nm.

1.2.5 Plasmid activity detection of the recombinant human-specific iNOS-luciferase reporter  The human iNOS-dependent luciferase reporter was kindly provided by Powell[5]. The human iNOS promoter fragment contains the unique human enhancer region which includes four nuclear factor kappa-B (NF-κB) binding sites critical for transcriptional regulation of iNOS[5]. A549 cells were seeded onto six-well plates and co-transfected with 1.0 µg of firefly-luciferase reporter plasmid and 0.1 µg of renilla-luciferase transfection control (pH-R-CMV; Promega, Southampton, UK) using Lipofectamine™ Plus Reagents. After 24 h transfection, the cells were treated with reagents for 24 h. The cells were harvested, added with cell lysate and then centrifuged at 14 000 X g for 5 min at 4 °C. Luciferase activity was measured with the Dual-Luciferase® Assay System according to the manufacturer's instructions by Lumat 9507.

1.2.6 Western blot  After 24 h treatment as described above, cell lysates were prepared by adding 500 µL lysis buffer (20 mmol/L pH 7.5 Tris-HCl, 150 mmol/L NaCl, 1% Nonidet P-40) containing 1% aprotinin and 1 mmol/L phenylmethylsulfonyl fluoride. The lysates were cleared by centrifugation and the protein concentration was determined by the DC Protein Assay (Bio-Rad, Hercules, CA, USA). Aliquots of the protein extracts (20 µg of protein each) were separated by 8.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, USA), and blocked with 5% nonfat milk in Tris buffered saline-Tween buffer (20 mmol/L pH 7.4 Tris-hydrochloric acid, 135 mmol/L sodium chloride, 0.1% Tween) for 1.5 h at room temperature, then incubated with the appropriate antibody (1:500) overnight at 4 °C, and then incubated with horseradish peroxidase-conjugated secondary antibody (1:5000) for 30 min at room temperature. After extensive washing, immunoreactive proteins were detected with an Enhanced Chemiluminescence Detection System (ECL; Amersham Biosciences Corp., Piscataway, NJ, USA). The following primary antibodies were used: monoclonal antibodies for iNOS, COX-2 and β-actin from transduction laboratory.

1.3 Statistical analysis  Data are presented as mean±standard deviation. Statistical analysis was performed with SPSS 10.0 statistical software. The comparisons among groups were performed with one-way analysis of variance or factorial design analysis of variance, and comparisons between groups were analyzed by LSD-t test. Statistical significance in all analyses was considered when P<0.05.

2 Results

2.1 Inhibitory effects of FYN on iNOS gene expression and NO production  Based on previous results[41], 15% serum-containing FYN was selected for all subsequent experiments. After 24 h treatment with 1 µg/L TNF-α, iNOS expression was significantly induced compared to that without TNF-α treatment (P<0.01). Treatment with FYN for 24 h significantly inhibited TNF-α-induced increases in iNOS mRNA level and iNOS protein (P<0.01, P<0.01). See Figures 1 and 2. We further examined the effect of FYN on NO production by DAF-2, and found that FYN treatment significantly inhibited NO production (P<0.01). See Figure 3.

2.2 FYN decreases iNOS promoter transcriptional activity induced by TNF-α  We performed the dual-luciferase assay with a reporter vector containing the iNOS promoter in A549 cells. As shown in Figure 4, compared with the control group, there was no significant difference in luciferase reporter activity in the FYN alone group. Upon stimulation with TNF-α, the luciferase reporter activity was significantly increased (P<0.01), and FYN significantly inhibited the iNOS promoter transcriptional activity induced by TNF-α (P<0.01). See Figure 4.

<table>
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<th>Primer sequence</th>
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<td>iNOS</td>
<td>5'-ATCCCGAAACGCTACACTT-3'</td>
<td>301</td>
</tr>
<tr>
<td></td>
<td>5'-AATCCACAATCGCTCACA-3'</td>
<td></td>
</tr>
<tr>
<td>COX-2</td>
<td>5'-CGTGATCCGCCCTGTCGTTG-3'</td>
<td>279</td>
</tr>
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<td></td>
<td>5'-ACTTGGCTCTGATATGTCGTTCTT-3'</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>5'-AAGTACTCGTGATGGATCGG-3'</td>
<td>547</td>
</tr>
<tr>
<td></td>
<td>5'-TCAAGTTGGGACAAAAAG-3'</td>
<td></td>
</tr>
</tbody>
</table>

iNOS: inducible nitric oxide synthase; COX-2: cyclooxygenase-2; PCR: polymerase chain reaction.
Figure 1  Inhibitory effect of FYN on increase in iNOS mRNA induced by TNF-α in A549 cells
The cells were incubated for 24 h. iNOS mRNA was determined by real-time polymerase chain reaction. Data are expressed as mean ± standard deviation; n=3; ** P<0.01, vs control group; △△ P<0.01, vs TNF-α group.

a: Control group; b: TNF-α group; c: FYN alone group (15% serum containing FYN); d: FYN plus TNF-α group. FYN: Feiyanning Decoction; iNOS: inducible nitric oxide synthase; TNF-α: tumor necrosis factor-α; A549: human lung adenocarcinoma epithelial cell line.

Figure 2  Inhibitory effect of FYN on increase in iNOS expression induced by TNF-α in A549 cells
The cells were incubated for 24 h. iNOS expression was determined by Western blot. Data are expressed as mean ± standard deviation; n=3; ** P<0.01, vs control group; △△ P<0.01, vs TNF-α group.

a: Control group; b: TNF-α (1 μg/L) group; c: FYN alone group (15% serum containing FYN); d: FYN plus TNF-α group. FYN: Feiyanning Decoction; iNOS: inducible nitric oxide synthase; TNF-α: tumor necrosis factor-α; A549: human lung adenocarcinoma epithelial cell line.

2.3 Inhibitory effect of FYN on COX-2 mRNA level
Treatment of FYN also affected COX-2 gene expression in the presence of TNF-α. Cells incubated with 1 μg/L TNF-α significantly induced COX-2 mRNA expression compared to those without TNF-α treatment, while treatment with FYN for 24 h repressed the increase in COX-2 mRNA level induced by TNF-α (P<0.01). See Figure 5.

Figure 3  Inhibitory effect of FYN on increase in NO production induced by TNF-α in A549 cells
The cells were incubated for 24 h. The DAF-2 assay was performed. Data are expressed as mean ± standard deviation; n=3; ** P<0.01, vs control group; △△ P<0.01, vs TNF-α group.

a: Control group; b: TNF-α group; c: FYN alone group (15% serum containing FYN); d: FYN plus TNF-α group. FYN: Feiyanning Decoction; NO: nitric oxide; TNF-α: tumor necrosis factor-α; A549: human lung adenocarcinoma epithelial cell line.

Figure 4  Effect of FYN on iNOS promoter in A549 cells
Cells plated in six-well plates were transfected with iNOS-luciferase plasmid. The cells were treated for 24 h after transfection, and then luciferase activity in the cell lysates was measured. Data are expressed as mean ± standard deviation; n=3; ** P<0.01, vs control group; △△ P<0.01, vs TNF-α group.

a: Control group; b: TNF-α (1 μg/L) group; c: FYN alone group (15% serum containing FYN); d: FYN plus TNF-α group. FYN: Feiyanning Decoction; iNOS: inducible nitric oxide synthase; TNF-α: tumor necrosis factor-α; A549: human lung adenocarcinoma epithelial cell line.

Figure 5  Inhibitory effect of FYN on increase in COX-2 mRNA induced by TNF-α in A549 cells
The cells were incubated for 24 h. COX-2 mRNA was determined by real-time polymerase chain reaction. Data are expressed as mean ± standard deviation; n=3; ** P<0.01, vs control group; △△ P<0.01, vs TNF-α group.

a: Control group; b: TNF-α group; c: FYN alone group (15% serum containing FYN); d: FYN plus TNF-α group. FYN: Feiyanning Decoction; COX-2: cyclooxygenase-2; TNF-α: tumor necrosis factor-α; A549: human lung adenocarcinoma epithelial cell line.
2.4 Inhibitory effect of FYN on COX-2 protein

As shown in Figure 6, cells incubated with 1 µg/L TNF-α significantly induced an increase in COX-2 protein compared to those without TNF-α treatment, while treatment with FYN for 24 h decreased COX-2 protein level ($P < 0.01$).

![Figure 6 Inhibitory effect of FYN on COX-2 expression induced by TNF-α in A549 cells](image)

The cells were incubated for 24 h. COX-2 expression was determined by Western blotting. Data are expressed as mean ± standard deviation; $n = 3$; $** P < 0.01$, vs control group; $\Delta \Delta P < 0.01$, vs TNF-α group.

a: Control group; b: TNF-α group; c: FYN alone group (15% serum containing FYN); d: FYN plus TNF-α group; FYN: Feiyanjing Decoction; COX-2: cyclooxygenase-2; TNF-α; tumor necrosis factor-α; A549: human lung adenocarcinoma epithelial cell line.

3 Discussion

Inflammation is a pathophysiological phenomenon known to be involved in numerous diseases, while it is notable that a considerable proportion of chronic inflammatory diseases overlap with the onset and development of cancer[10]. It has also been well documented that infection with microorganisms is closely related to inflammation-derived carcinogenesis[11]. Two of the most prominent are the production of NO by iNOS and the formation of prostaglandin by COX-2[12,13]. iNOS is a ubiquitous transcription factor that is activated by a variety of cytokines and mutagens, and is a key regulator in the process of cell survival and deterioration[14]. NF-κB, an oxidative-stress-responsive transcription factor, is activated and mediates cytokine-induced expression of iNOS in various cells[15]. Gene COX-2 is an NF-κB-inducible gene that plays important roles in carcinogenesis[16]. Both iNOS and COX-2 have drawn considerable attention for their critical functions in inflammation-related diseases[10,14]. They are also the new targets in drug design projects in cancer pharmacology and clinical trials[17].

FYN is a compound for nourishing qi and yin and diminishing stagnation by detoxification func-

4 Competing interests

The authors declare that they have no competing interests.

REFERENCES


肺岩宁方在人肺癌细胞系对肿瘤坏死因子α诱导的iNOS和COX-2表达的影响

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目的：探讨中药肺岩宁方含药血清对肿瘤坏死因子α(tumor necrosis factor-α，TNF-α)诱导的人肺癌细胞系A549诱导型一氧化氮合酶(inducible nitric oxide synthase，iNOS)和环氧合酶2(cyclooxygenase-2，COX-2)表达的影响。

方法：制备肺岩宁方含药血清，以肺癌细胞系A549为靶细胞，实时聚合酶链反应法及蛋白质免疫印迹法检测肺岩宁方含药血清对TNF-α诱导的iNOS及COX-2表达的影响；氨基乙酰乙酰氯素检测NO产物，人iNOS重组质粒用脂质体转染法导入A549细胞24 h后，肺癌宁方含药血清治疗24 h，采用双荧光素酶报告基因系统测定肺岩宁方含药血清对TNF-α诱导的细胞核iNOS转录活性的影响。

结果：与对照血清比较，TNF-α明显提高iNOS和COX-2的表达，与肺岩宁方含药血清共同作用后，显著抑制二者的表达(P<0.01，P<0.01)；肺岩宁方含药血清显著降低了A549细胞经iNOS重组质粒转染后的转录活性(P<0.01，P<0.01)及NO产物的产生。

结论：肺岩宁方含药血清抑制肺癌细胞的部分机制可能是抑制iNOS和COX-2基因的活性。

关键词：肺肿瘤；一氧化氮合酶；环氧合酶2；肿瘤坏死因子α；肺岩；复方；大鼠