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Luteolin inhibits the Nrf2 signaling pathway and tumor growth *in vivo*

Song Chian [a], Ruby Thapa [a], Zhexu Chi [a], Xiu Jun Wang [b], Xiuwen Tang [a,*]

[a] Department of Biochemistry and Genetics, School of Medicine, Zhejiang University, Hangzhou 310058, PR China
[b] Department of Pharmacology, School of Medicine, Zhejiang University, Hangzhou 310058, PR China

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ABSTRACT

Nuclear factor erythroid 2-related factor 2 (Nrf2) is over-expressed in many types of tumor, promotes tumor growth, and confers resistance to anticancer therapy. Hence, Nrf2 is regarded as a novel therapeutic target in cancer. Previously, we reported that luteolin is a strong inhibitor of Nrf2 *in vitro*. Here, we showed that luteolin reduced the constitutive expression of NAD(P)H quinone oxidoreductase 1 in mouse liver in a time- and dose-dependent manner. Further, luteolin inhibited the expression of antioxidant enzymes and glutathione transferases, decreasing the reduced glutathione in the liver of wild-type mice under both constitutive and butylated hydroxyanisole-induced conditions. In contrast, such distinct responses were not detected in *Nrf2*$^{-/-}$ mice. In addition, oral administration of luteolin, either alone or combined with intraperitoneal injection of the cytotoxic drug cisplatin, greatly inhibited the growth of xenograft tumors from non-small-cell lung cancer (NSCLC) cell line A549 cells grown subcutaneously in athymic nude mice. Cell proliferation, the expression of Nrf2, and antioxidant enzymes were all reduced in tumor xenograft tissues. Furthermore, luteolin enhanced the anti-cancer effect of cisplatin. Together, our findings demonstrated that luteolin inhibits the Nrf2 pathway *in vivo* and can serve as an adjuvant in the chemotherapy of NSCLC.

1. Introduction

The transcription factor Nrf2 was originally described as a master regulating protein of the intracellular antioxidant response through transcriptional activation of an array of genes involving conjugation/detoxification reactions (e.g. glutathione S-transferase), anti-oxidative responses (e.g. NADPH quinone oxidoreductase 1 (NQO1), heme oxygenase-1 (HO-1), and aldo–keto reductase family 1, member C1), the glutamate cysteine ligase catalytic subunit, and proteasome function (proteasome subunits). Therefore, the Nrf2 pathway is of great importance in cytoprotection by maintaining the cellular redox balance and providing an adaptive response to oxidative or electrophilic stress [1,2]. The major negative regulator of Nrf2 is Kelch-like ECH-associated protein 1 (Keap1), which mediates its proteasomal degradation in the cytoplasm [3–5]. Oxidative stress, as well as electrophilic and chemical inducers, can modify the reactive cysteines in Keap1 and cause the release of Nrf2 from the Keap1/Nrf2 complex or conformational changes in Keap1 that prevent Nrf2 from being degraded *via* the proteasomal pathway. As a result, Nrf2 accumulates and translocates to the nucleus, where it interacts with small Maf, and the heterodimer binds to the antioxidant response element sequences (ARE; 5′-NTGAG/CNNNGC-3′) in Nrf2 target genes, hence inducing their transcription [6–8]. Dysfunction of this pathway leads to many oxidative stress-related diseases including cancer. Recently, somatic mutations in Nrf2 and Keap1 have been reported in many cancers, including those in the lung [9], gall bladder [10], and head-and-neck [11]. These mutations lead to constitutive activation of the Nrf2 pathway, which enhances chemoresistance and cell proliferation [12,13]. Indeed, cancer cells and oncogenes hijack Nrf2 activity for malignant growth. Therefore, it is a rational strategy to discover small-molecule modulators of the Nrf2 pathway for cancer prevention and therapy [14].

Luteolin is a flavonoid that exists in food plants and vegetables [15]. Studies by Bagli et al. [16], Kim et al. [17], and Lopez-Lazaro [18] have shown that plants rich in luteolin have a wide range of biological actions ranging from antioxidant, anti-inflammatory, and anti-allergy to anticancer effects. Recently, we reported that luteolin is an Nrf2 inhibitor that enhances Nrf2 mRNA degradation, leads to reduced expression of the ARE-gene battery, and sensitizes the A549 non-small-cell lung cancer (NSCLC) cell line to therapeutic drugs [19]. However, the effect of luteolin on the Nrf2 signaling pathway *in vivo* requires further investigation. In this report, using the Nrf2$^{-/-}$ mouse as a control, we showed that luteolin negatively regulated the Nrf2 target genes *in vivo*. Furthermore, in a nude mouse model we demonstrated that luteolin inhibited tumor

* Corresponding author. Address: PO Box 18, School of Medicine, Zhejiang University, Hangzhou 310058, PR China. Fax: +86 571 88208266.
E-mail address: xiuwentang@zju.edu.cn (X. Tang).

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growth and enhanced the anti-cancer effect of cisplatin, with reduced expression of Nrf2 and its target genes. Therefore, we provide evidence that targeting Nrf2 is a new strategy for sensitizing NSCLC to anti-cancer drugs.

2. Materials and methods

2.1. Chemicals and cell lines

Unless otherwise stated, all chemicals were from Sigma–Aldrich Co., Ltd (Shanghai, China), and all antibodies were from Santa Cruz Biotechnology (Shanghai, China). A549 (human NSCLC) cell lines were purchased from ATCC (China). The AKR1C and Gstm1 antibodies were kindly provided by Professor John Hayes (University of Dundee, Scotland). Luteolin was obtained from Sky Herb Technologies Co. Ltd (Hangzhou, China). Complete EDTA-free protease inhibitor tablets were from Roche Diagnostics Ltd (Lewes, UK).

2.2. Animals

The *Nrf2*$^{-/-}$ mice (C57BL/6) were from Dr. Masayuki Yamamoto (University of Tsukuba, Japan). The C57BL/6 mice were from the Shanghai Laboratory Animals Co. Ltd (Shanghai, China). Six-week-old male C57BL/6 *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ mice were used. Luteolin (in 0.5% carboxymethylcellulose, CMC) and control (0.5% CMC), and butylated hydroxyanisole (BHA; 300 mg/kg) or control in corn oil, were delivered by intragastric gavage daily for the entire study. Mice were housed in a laminar air-flow cabinet under specific pathogen-free conditions. They were routinely fed and given free access to water. All animal procedures were performed with the approval of the Laboratory Animals Ethics Committee of Zhejiang University.

For the mouse xenograft model, 6-week-old (20–22 g) female athymic nu/nu nude mice (Shanghai Laboratory Animals Co. Ltd) were subcutaneously inoculated with A549 tumor cells (1×10^7 - cells) into the right flank. Tumors were serially measured thrice per week using Vernier calipers, and tumor volume was calculated using the formula $V = \pi/6 \times$ larger diameter $\times$ (smaller diameter)^2, as described by Lopez-Lazaro [18]. The mean and standard error (SE) were then calculated for each experimental group for each time point. Once the tumor size reached 60 mm^3, mice were randomly allocated into four groups ($n = 6$) and treated with normal saline, cisplatin only (5 mg/kg), luteolin only (40 mg/kg), or a combination of cisplatin (5 mg/kg) and luteolin (40 mg/kg). Luteolin and control (0.5% CMC) were delivered by intra-gastric gavage thrice per week for 35 days (15 times in total). Mice in the cisplatin and combination groups were given cisplatin by intraperitoneal injection twice per week, a total of six times. The health of the animals was monitored by measuring body weight. At the end of the experiments the animals were sacrificed and tumors were dissected out and weighed.

2.3. Histopathology

Tumor samples were collected immediately after the animals were killed and placed in 4% paraformaldehyde or frozen in liquid nitrogen for future analysis of protein expression. Tumor sections were stained with hematoxylin and eosin (H&E) or for immunohistochemistry (IHC) using an Envision kit (Dako Corporation, Carpinteria, CA). For negative controls, sections were incubated with rabbit IgG in place of the primary antibody. Images were captured under a light microscope (Olympus BX41, Shanghai, China) at 400× magnification. Image Pro Plus6.0 (Media Cybernetics Inc, Shanghai, China) was used to calculate the staining intensity. Three microscopic fields in tumor tissues were randomly selected and the integral optical density (IOD) of Ki-67, Nrf2, NQO-1, and HO-1 was calculated, and this was considered as the expression level. Higher IOD values represented higher antigen expression, and *vice versa* [18].

2.4. Western blot analysis

Samples were prepared as described by Chanas et al. [20]. The protein samples were subjected to SDS–PAGE and immunoblotting was performed using the standard protocol. Band intensity was scanned on an Odyssey scanner (Li-Cor Biosciences, CA, USA) and the resulting images were analyzed densitometrically using Odyssey infrared imaging system software. The relative levels of protein were calculated by quantification of band intensity and normalized to actin [19].

2.5. Measurement of reduced glutathione

Reduced glutathione was measured as described by Kamencic et al. [21].

2.6. Statistical analysis

Statistical comparisons were performed using unpaired Student's *t*-tests. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Luteolin inhibited the Nrf2 signaling pathway in mouse liver

3.1.1. Luteolin inhibited NQO1 expression in vivo in a dose- and time-dependent manner

As previous studies showed that luteolin inhibits the Nrf2 pathway in cultured tumor cells, we sought to determine whether this effect occurs *in vivo*. The expression of NQO1, a readout of Nrf2 activation, was investigated in C57BL/6 mice. The mice were given either 0.5% CMC containing different amounts of luteolin (10, 40, or 80 mg/kg) or 0.5% CMC alone for 14 days, and NQO1 protein expression in the liver was assessed by Western blot. A significant reduction of NQO-1 expression was found after luteolin treatment (Fig. 1A). The NQO1 expression declined 25% with 10 mg/kg, 50% with 40 mg/kg, and 44% with 80 mg/kg luteolin compared with the CMC control. The average body weights of mice, with or without luteolin treatment, were comparable during the experimental period (data not shown), and the luteolin-treated mice did not show any signs of toxicity or abnormal behavior (data not shown). These findings demonstrated that administration of luteolin at the concentrations used did not cause gross toxicity. We further investigated the time-effects of luteolin (40 mg/kg) on the expression of NQO1. Compared with the CMC control, the constitutive expression of NQO1 decreased in the liver by 5%, 25%, 45%, 47%, and 41% after 3, 7, 10, 14, and 21 days of treatment, respectively (Fig. 1B).

3.1.2. Luteolin inhibited both constitutive and inducible expression of Nrf2-regulated genes in vivo

To assess its inhibitory effect on the Nrf2 pathway *in vivo* in more detail, luteolin (40 mg/kg) was given to *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ mice. After 14 days of treatment, the livers were harvested, and the levels of expression of Nrf2 target genes were evaluated by Western blot. Luteolin treatment decreased the protein level of NQO1 by 38%, AKR1C by 28%, HO-1 by 27%, and GSTm1 by 38% compared with the CMC control in the *Nrf2*$^{+/+}$ mice. But it had no effect in the *Nrf2*$^{-/-}$ mice (Fig. 2A).