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

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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

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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).

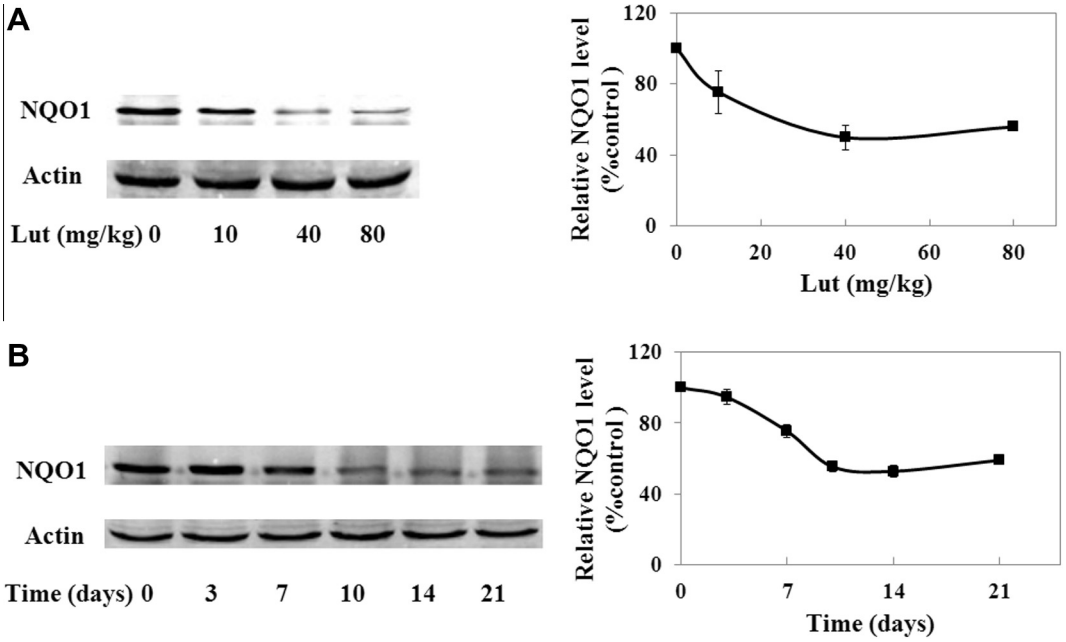

Fig. 1. Luteolin inhibited NQO1 expression *in vivo* in a dose- and time-dependent manner. (A) Dose-dependent changes in the level of NQO1 protein in the liver of C57BL/6 mice gavaged with either CMC or different doses of luteolin daily for 14 days. (B) Changes in the protein level of constitutive NQO1 in the liver of C57BL/6 mice gavaged with luteolin (40 mg/kg) daily for different times. Left panels: Western blots; right panels: NQO1 normalized to actin. Experiments were repeated three times.

Luteolin also strongly inhibited the inducible level of Nrf2 target genes in the liver (Fig. 2B). After treating the mice with the Nrf2 inducer BHA, the expression of NQO1 in the liver increased by 2-fold, AKR1C by 1.6-fold, HO-1 by 2.5-fold, and GSTm1 by 2.4-fold. Luteolin attenuated the BHA-induced expression of these proteins in the liver by ~40% after co-treating the mice with BHA and luteolin. In contrast, such conspicuous responses were not detected in the *Nrf2*$^{-/-}$mice (Fig. 2B).

Next, we further determined the effect of luteolin on the level of reduced glutathione (GSH) in tissues. When comparing luteolin treatment with CMC control, the GSH level was reduced in the liver by 31% (Fig. 2C). After treating the mice with the Nrf2 inducer BHA, the level of reduced GSH in the liver increased by 2.2-fold. Luteolin abolished this increase when co-administered with BHA (Fig. 2D). Although the levels of NQO1, HO1, and Gstm1 were markedly reduced as a result of Nrf2 knockout, the expression of these genes was not altered significantly by luteolin, BHA, or both (Fig. 2A and B). Accordingly, the level of reduced GSH markedly declined in the liver as a result of Nrf2 knockout, but was not altered significantly by luteolin, BHA, or both (Fig. 2C and D). Thus, our data indicated that luteolin inhibits Nrf2 *in vivo*, and Nrf2 is an important target of luteolin.

3.2. Luteolin inhibited tumor growth and the Nrf2 pathway in a mouse xenograft model

The study of mice showed that luteolin down-regulated Nrf2 *in vivo*. We further investigated the effect of luteolin on tumor growth and its inhibition of the Nrf2 pathway in a xenograft tumor model. Nude mice were inoculated with human NSCLC A549 cells, and tumor-bearing mice were randomly allocated into four groups (6/group): control, cisplatin, luteolin, and both cisplatin and luteolin. Both luteolin and cisplatin significantly inhibited tumor growth (Fig. 3A). Compared with the untreated mice, the average body weights decreased and were reduced by 18% after 35 days of cisplatin treatment. Surprisingly, the average body weights were comparable to the untreated mice in those treated with either luteolin alone or co-administration of luteolin together with cisplatin (Fig. 3B). The total tumor weight was reduced by 55% in the luteolin and 47% in the cisplatin group compared with the CMC control. Significantly, luteolin enhanced the toxic effect of cisplatin and the combination treatment resulted in a 70% reduction of tumor weight compared with control (Fig. 3C).

To further address the mechanisms underlying the inhibition of tumor progression and proliferation, A549 xenograft tumors were analyzed by IHC with Ki67 staining. The percentage of Ki67-positive cells was 41% in the control group, 31% in the luteolin group, 32% in the cisplatin group, and 25% in the combination group (Fig. 3D). The data showed that the proliferative activity was lower in all treated groups than in controls ($p < 0.01$). Furthermore, the tumor xenografts were subjected to pathological examination and H&E-stained sections revealed little difference in cellular atypia and karyokinesis among the groups (Fig. 3D).

Immunoblotting of tumor xenografts revealed that the expression of components of the Nrf2 pathway was inhibited to asimilar extent in theluteolin and the combination treatments. In comparison with the control, Nrf2 expression was inhibited to 60%, NQO1 to 72%, AKR1C to 55% and HO-1 to 58% in the luteolin and combination treatments, whereas there was no significant reduction in the cisplatin group (Fig. 4A). Furthermore, the A549 xenograft tumors were analyzed by IHC with Nrf2, NQO-1, and HO-1staining. Compared with the control, Nrf2 expression was inhibited to 36% in the luteolin group and 30% in the combination group; the expression of NQO1 declined to 75% in the luteolin group and 78% in the combination group; and the expression of HO-1 was inhibited to 67% in the luteolin group and 52% in the combination group, which confirmed that luteolin significantly inhibited the Nrf2 pathway in both the luteolin and the combination groups, while cisplatin had no effect (Fig. 4B).

4. Discussion

Since the transcription factor Nrf2 was originally identified as a master regulator of the intracellular antioxidant response, it has been proposed that Nrf2 is of great importance in protection

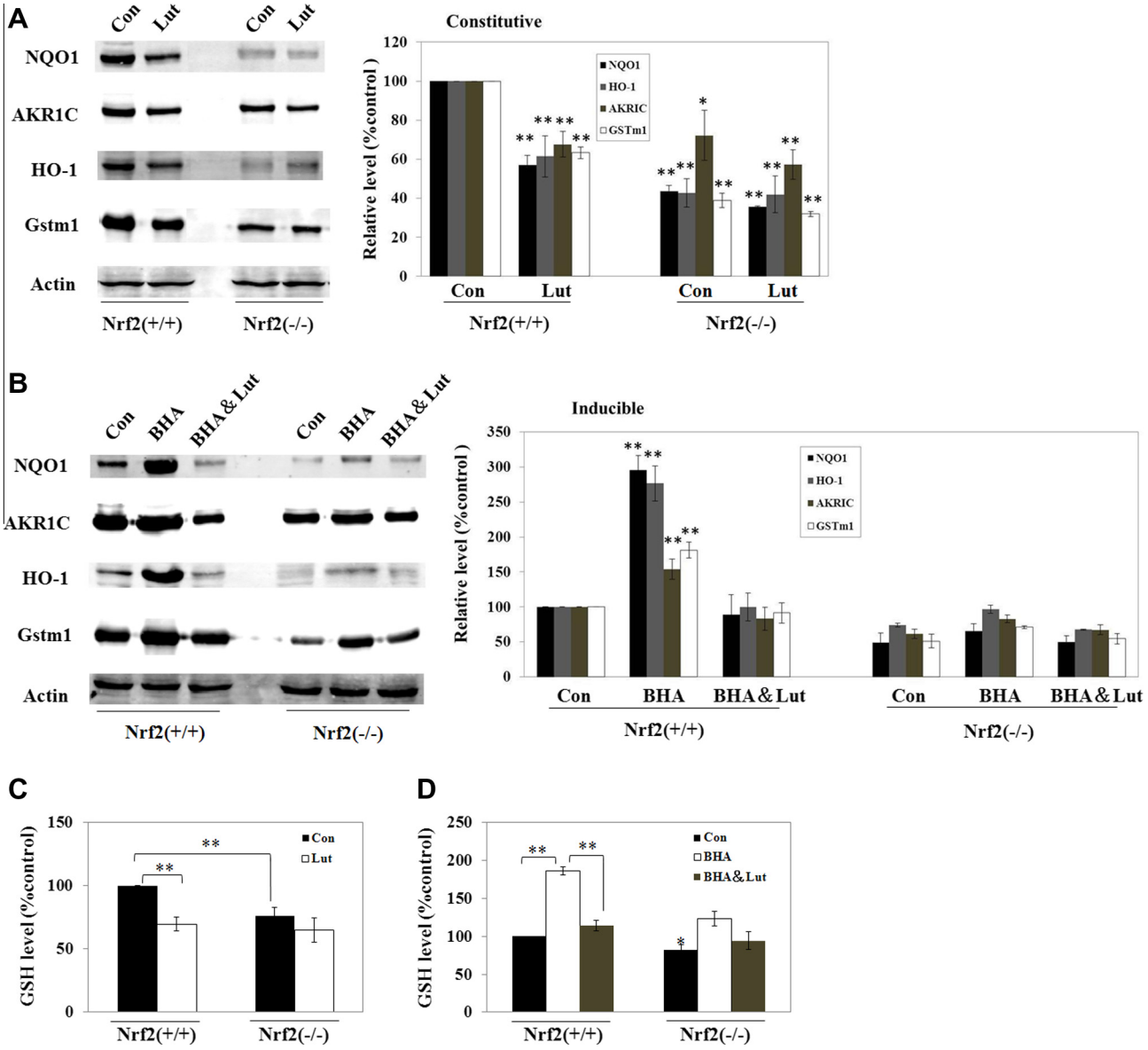

Fig. 2. Luteolin inhibited the Nrf2/ARE pathway *in vivo*. (A and B) Luteolin inhibited both the basal and the inducible expression of Nrf2 target genes *in vivo*. Cytosol from the liver of wild-type and KO mice were analyzed by SDS–PAGE and the expression of NQO1, AKR1c, HO1, GSTα1/2, GSTm1, and actin were assessed by Western blot. (A) Male $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice were given either CMC or luteolin (40 mg/kg) orally each day for 14 days. (B) Male $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice were given either corn oil or the synthetic antioxidant BHA (300 mg/kg) or combined BHA (300 mg/kg) and luteolin (40 mg/kg) (BHA&Lut) orally each day for 14 days. NQO1, AKR1C, HO-1, and Gstm1 normalized to actin. The immunoblots are typical of at least three replicates. (C and D) Luteolin reduced the level of glutathione *in vivo*. (C) Male $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice were given either CMC or luteolin (40 mg/kg) orally each day for 14 days. D. Male $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice were given either corn oil or the synthetic antioxidant BHA (300 mg/kg) or combined BHA (300 mg/kg) and luteolin (40 mg/kg) (BHA&Lut) orally each day for 14 days. Cytosol from the liver of $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice were assayed for GSH levels. The control (CMC or corn oil in wild-type) was set at 100%. Values are mean ± SD. Results are from at least three separate experiments ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$(Student's *t*-test).

against diseases caused by oxidative stress, including cancer. There has been great interest in identify small molecules to activate the Nrf2-dependent defense response for disease prevention. However, it is now well-known that uncontrolled Nrf2 activation enhances tumorigenesis and drug resistance during chemo- and radio-therapy. Therefore, pharmacological inhibitors of the Nrf2 signaling pathway could be of therapeutic value. Indeed, we identified luteolin as an Nrf2 inhibitor using an ARE-reporter assay. Our previous work showed that luteolin renders cancer cells susceptible to anticancer drugs *in vitro* [19]. In NSCLC A549 cells, mutation of Keap1 in the Kelch domain (G333C) that weakens its binding with Nrf2 leads to constitutive activation of Nrf2 in this cell line [22]. Luteolin inhibits the expression of Nrf2 target genes in a redox-independent manner in A549 cells [19]. Luteolin enhanced Nrf2 mRNA degradation, leading to a dramatic decrease in Nrf2 at both the mRNA and protein levels, a reduction in Nrf2 binding to AREs, diminished expression of ARE-driven genes, and depletion of reduced glutathione. We further investigated the effects of luteolin on Nrf2 target genes *in vivo* in an animal model. We report the following new findings: (a) luteolin specifically inhibited the Nrf2 pathway in mouse liver without apparent toxicity; (b) luteolin significantly inhibited the growth of human NSCLC carcinoma xenografts in nude mice; and (c) the combination of luteolin and cisplatin was more effective in inhibiting tumor cell growth than either luteolin or cisplatin alone. Luteolin-mediated sensitization to cisplatin could depend on its inhibition of the Nrf2 pathway through down regulation of the expression of Nrf2, and antioxidant and xenobiotic-metabolizing enzymes in xenograft tumor cells.

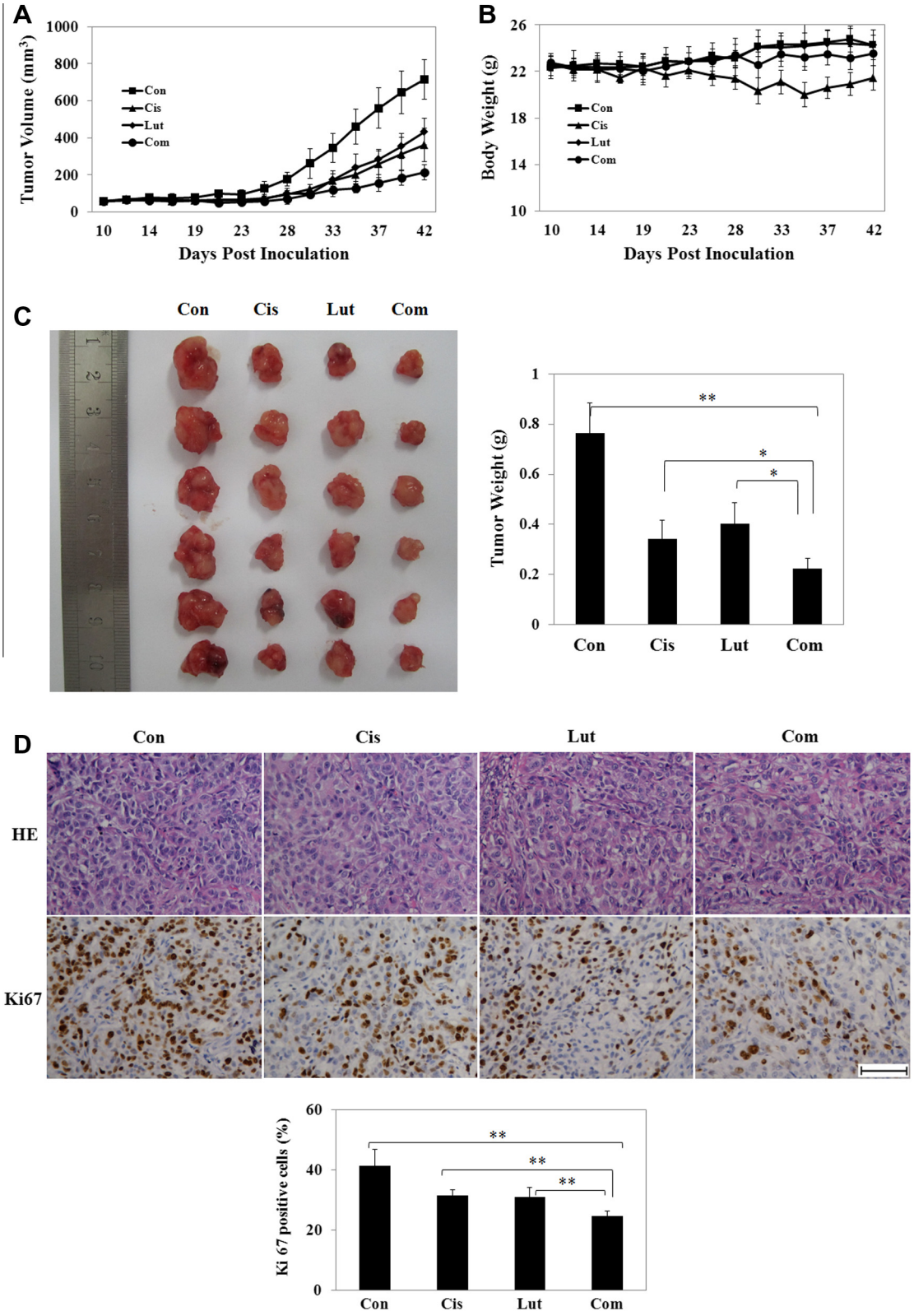

Fig. 3. Luteolin sensitized xenografts to cisplatin treatment. Nude mice (6/group) were injected with A549 cells. Once tumor size reached 60 mm^3, mice were given normal saline (control), cisplatin (5 mg/kg, i.p.), luteolin (40 mg/kg, gavage), or both every other day for 32 days. At the end of the experiments (42 days), mice were sacrificed. (A) Tumor growth curves for A549 xenograft tumors. (B) Body weight curves for mice with different treatments. There was no significant loss of body weight (<20%) in mice treated with cisplatin. (C) Excised xenograft tumors (left panel) and weights (right panel) at the end of the experiment. (D) Histopathology of xenograft tumors stained with H&E and anti-Ki67 antibody. Original magnification 100×; scale bar, 10 μm. Ki67-positive cells were countedin representative fields. Columns, mean ($n = 6$); bars, SD; $^{*}p < 0.05$, $^{**}p < 0.01$(Student's *t*-test).

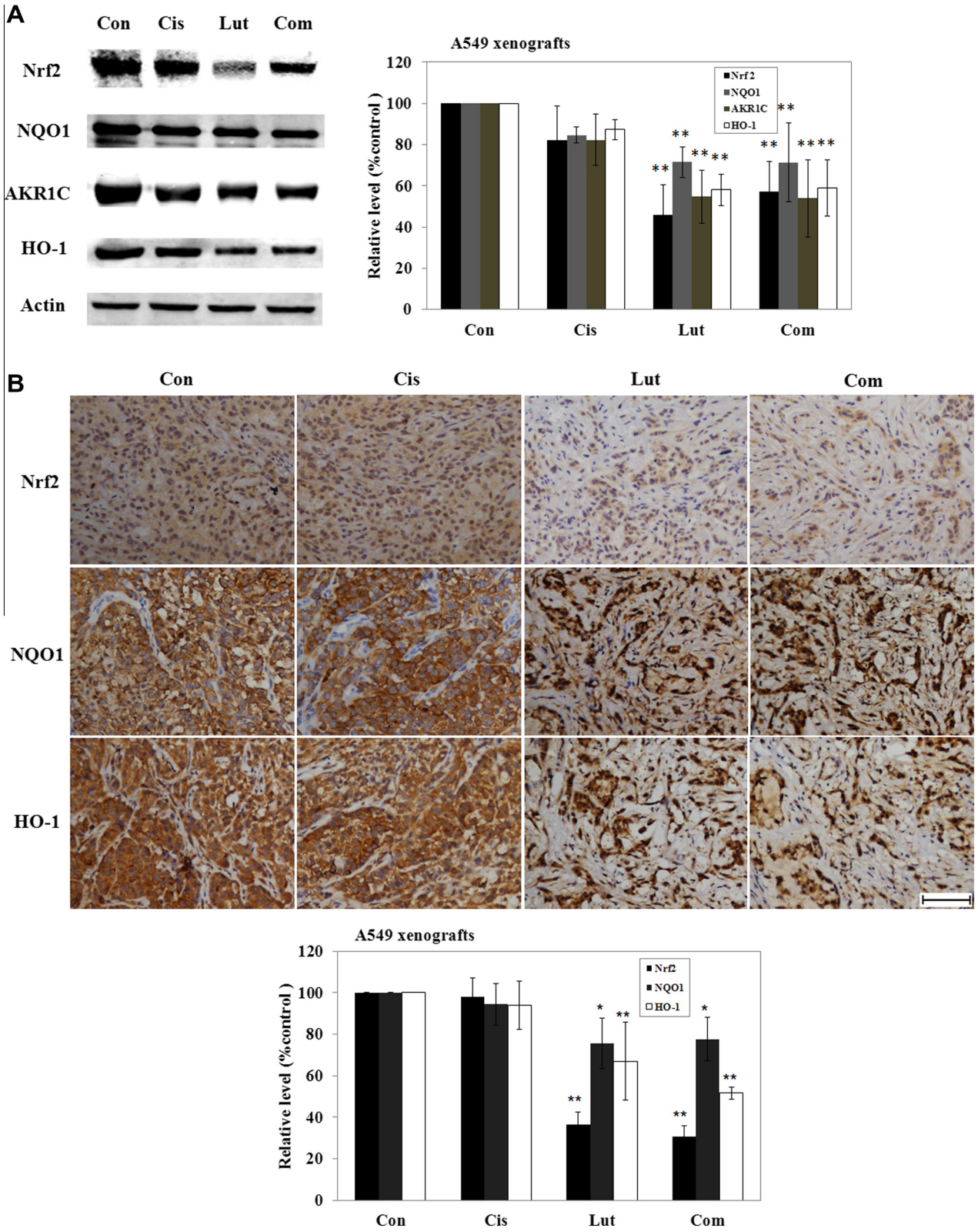

Fig. 4. Luteolin inhibited the Nrf2 pathway in xenograft tumors. (A) Immunoblots of the expression of the Nrf2 pathway components in xenografts. Nrf2, NQO1, AKR1C, and HO-1 normalized to actin. The immunoblots are typical of six replicates. (B) Histopathology of xenograft tumors. Tumor sections stained with anti-Nrf2, anti-NQO1, and anti-HO-1 antibodies. Original magnification 100×; scale bar, 10 μm. The expression levels of Nrf2, NQO1, and HO-1 quantified by IOD analysis; the relative Nrf2 and NQO1 and HO-1 staining were quantified as percentages of control. Columns, mean ($n = 6$); bars, SD; $^{*}p < 0.05$, $^{**}p < 0.01$(Student's t-test).

These findings indicated that the identification of Nrf2 inhibitors is important [23,24]. However, achieving specificity is the greatest challenge in the development of inhibitors targeting Nrf2 because it belongs to the CNC protein family that includes NF-E2 p45, Nrf1, Nrf3, and Bach2, and Motohashi has shown that all members share many properties [25]. In addition, achieving delivery-specificity is an important issue. The systemic inhibition of Nrf2 could worsen the side-effects of chemo- and radio-therapy;

Study by Satoh et al. showed that Nrf2-deficiency in bone marrow cells aggravates metastasis [26]. Surprisingly, luteolin was non-toxic in mice in our study, implying that its effect on the Nrf2 pathway may be cell-type dependent. Indeed, Lin et al. demonstrated that luteolin has an opposite effect in PC12 cells, activating Nrf2 and ARE-dependent luciferase activity [15]. Most recently, Zhang et al. showed that the Nrf2-activating and antioxidant properties of luteolin protect neurons against ischemia-induced injury [27]. Therefore, the effects of luteolin on the Nrf2 pathway are cell-type specific and may involve multiple mechanisms.

In conclusion, we have shown that luteolin inhibits Nrf2 in mouse liver and in xenografted tumors. Luteolin, a dietary flavonoid, significantly enhances the therapeutic effect of cisplatin both in animal models and in cell lines. Our results strongly suggest that luteolin may serve as a chemosensitizer in NSCLC cells with Nrf2 overexpression.

Conflict of interest

The authors have declared that there is no conflict interest.

Acknowledgments

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