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Flavone inhibits nitric oxide synthase (NOS) activity, nitric oxide production and protein S-nitrosylation in breast cancer cells

Wenzhen Zhu [a], Bingwu Yang [a], Huiling Fu [a], Long Ma [a], Tingting Liu [a], Rongfei Chai [a], Zhaodi Zheng [a], Qunye Zhang [b, *], Guorong Li [a, *]

[a] Shandong Provincial Key Laboratory of Animal Resistant Biology, School of Life Sciences, Shandong Normal University, Jinan 250014, China
[b] Key Laboratory of Cardiovascular Remodeling and Function Research Chinese Ministry of Education and Ministry of Public Health, Qilu Hospital, Shandong University, Jinan, Shandong, China

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ABSTRACT

As the core structure of flavonoids, flavone has been proved to possess anticancer effects. Flavone's growth inhibitory functions are related to NO. NO is synthesized by nitric oxide synthase (NOS), and generally increased in a variety of cancer cells. NO regulates multiple cellular responses by S-nitrosylation. In this study, we explored flavone-induced regulations on nitric oxide (NO)-related cellular processes in breast cancer cells. Our results showed that, flavone suppresses breast cancer cell proliferation and induces apoptosis. Flavone restrains NO synthesis by does-dependent inhibiting NOS enzymatic activity. The decrease of NO generation was detected by fluorescence microscopy and flow cytometry. Flavone-induced inhibitory effect on NOS activity is dependent on intact cell structure. For the NO-induced protein modification, flavone treatment significantly down-regulated protein S-nitrosylation, which was detected by "Biotin-switch" method. The present study provides a novel, NO-related mechanism for the anticancer function of flavone.

1. Introduction

Nitric oxide (NO) is an endogenous, short-lived, pleiotropic signaling molecule, which is synthesized from L-arginine and oxygen by nitric oxide synthase (NOS). There are mainly three isoforms of NO synthase: endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS) [1,2]. NO level and NOS activity have been found increased in multiple kinds of cancers, including breast cancer, lung cancer, gastric cancer, bladder cancer and oral cavity cancer [3–5]. NO promotes the hepatitis B virus X protein (HBx)-induced hepatocellular carcinoma by up-regulating HBx gene expression and JNK signaling pathway [6]. NO and its product peroxynitrite promote inflammation-related carcinogenesis by inducing DNA damage, facilitating formation of DNA lesion 8-nitroguanine and restraining DNA repair system. NO promotes cell proliferation by up-regulating endogenous basic fibroblast growth factor (bFGF), mitogen-activated protein kinase (MAPK) pathway and induction of heat-shock protein 90, 70 and 32. NO inhibits cell apoptosis with decreasing release of cytochrome C, suppressing caspase activation and stabilizing Bcl-2 by inducing cysteine S-nitrosylation [2,5,7–10].

Nitric oxide regulates a lot of cellular responses by S-nitrosylation. Protein S-nitrosylation is a reversible post-translational modification which occurs on protein cysteine residues, by attaching an NO moiety with a reactive cysteine thiol group to form an S-nitrosothiol (SNO). Experimental evidence has indicated that the dysregulation of S-nitrosylation involves in numerous cancer-related pathological events, such as tumor origination, development, metastasis and treatment resistance [11–14]. S-nitrosylation on the catalytic-site cysteines of caspase-3 inhibits its activity, and then suppresses apoptosis [8,15]. For the antiapoptotic protein Bcl-2, S-nitrosylation on the cysteine residues (Cys158 and Cys229) inhibits ubiquitination and subsequent proteasomal degradation, thus enhances Bcl-2 stabilization [7,16]. The activation of tumor suppressor gene PTEN is inhibited by S-nitrosylation, which could be prevented by S-sulfhydration [17].

Flavonoids are widespread in nature, regarded as safe and easily obtainable, because they can be extracted from natural plants, and many of them are part of the daily human diet [18,19]. Flavonoids

* Corresponding authors.
E-mail addresses: wz.zhangqy@sdu.edu.cn (Q. Zhang), grli@sdnu.edu.cn (G. Li).

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have been demonstrated to regulate NO-related cellular processes. Quercetin inhibits NO accumulation and activity of iNOS in SAS human oral cancer cells [20], cholangiocarcinoma (CCA) cells [21], and RINm5F rat insulinoma cells [22]. Acacetin suppresses transcriptional activities of Stat-1 and Stat-3 by inhibiting Tyr phosphorylations, and then reduces expressions of downstream targets iNOS and eNOS in human umbilical vein endothelial cells as well as human lung cancer and prostate cancer cells [23]. Epigallocatechin-3-gallate (EGCG) suppresses NO production and iNOS activity in cholangiocarcinoma (CCA) cells and murine mammary carcinoma cells, and reduces the LPS-induced S-nitrosylation of superoxide dismutase 2 (SOD2), seroxiredoxin (PRDX) and ubiquitin carboxyl-terminal hydrolase 14 (USP14) in murine BV-2 microglial cells [21,24,25]. Puerarin inhibits oxidative stress and protein S-nitrosylation in the rat diabetic kidneys [26].

Flavone, 2-phenyl-4H-1-benzopyran-4-one, is the core structure of flavonoids, which has been shown to possess antitumor function in human colon and breast cancer cells [27–29]. Some results implied that, maybe flavone's growth inhibitory effects are related to NO. In HT-29 human colon cancer cells, flavone induces cell apoptosis, activation of caspase-3-like and release of cytochrome C, which are all repressed by NO donor SNP, whereas the same concentration SNP treatment only do not cause any change in caspase-3-like activity [30]. In murine J774 macrophages and rat hepatocyte, flavone inhibits the LPS-induced NO production, iNOS expression and activation of nuclear factor-κB (NF-κB), which is an important transcription factor for iNOS [31,32]. But, in cancer cells, there is still no definite experimental evidence about flavone's effect on NO.

In this study, we explored the flavone-induced anticancer effect and regulations on NO-related mechanism in MCF-7 and MDA-MB-231 breast cancer cells. Flavone inhibited cell proliferation and caused apoptosis by concentration- and time-dependent manner. We found flavone decreased NO production by inhibiting NOS activity, and then down-regulated the level of protein S-nitrosylation, which is a post-translational modification mediated by NO. In our knowledge, this is the first specific report focused on flavone-induced regulations on NO-related cellular processes and S-nitrosylation in cancer cells.

2. Materials and methods

2.1. Cell culture

The human breast cancer cell lines MCF-7 and MDA-MB-231 were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere containing 5% CO_2–95% air.

2.2. MTT assay

MTT assay is based on the conversion of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Amresco, US) to formazan crystals by mitochondrial dehydrogenases. Briefly, MCF-7 cells were seeded in 96-well plates at a density of 2×10^4 cells per well for 18 h. Cells were treated with or without flavone under various concentrations (0, 100, 150, 200, 250 μM) for diverse periods of time (24, 48, 72 and 96 h). Then MTT solution (5 mg/mL) was added (20 μL per well). After incubation at 37 °C for 4 h, the formazan crystals were dissolved with 150 μL dimethyl sulfoxide (DMSO, Amresco, US). Finally, the absorbance was measured at 492 nm by an enzyme-linked immunosorbent assay reader (Awareness, US).

2.3. Hoechst/propidium iodide nuclear staining and fluorescence microscopy

MCF-7 cells were treated with or without flavone (200 μM) for 24, 48 and 72 h. The staining was performed using Apoptosis and Necrosis Assay Kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Cells were stained with 10 ng/mL Hoechst 33342 and 10 ng/mL propidium iodide (PI) for 25 min at 0 °C in dark. Finally, the nuclei of apoptotic cells were observed by fluorescence microscope. This method differentiates the normal live cells (stained light blue), apoptotic cells (stained bright blue) and necrotics cells (stained red). A minimum of 500 cells were counted.

2.4. Assay of NOS enzymatic activity

NOS enzymatic activity in MCF-7 and MDA-MB-231 cells was measured according to the manufacturer's specification with Nitric Oxide Synthase Assay Kit (Beyotime, China). Fluorescence was measured with a fluorescence microplate reader at excitation of 495 nm and emission of 515 nm.

2.5. Assay of NOS enzymatic activity in cell lysate

MCF-7 cells were lysed by “Cell and Tissue Lysis Buffer for Nitric Oxide Assay” (Beyotime, China), and centrifuged at 12,000 g for 4 min. The supernatant collected was incubated with 50, 100 and 200 μM flavone at 37 °C for 1, 3 and 24 h. NOS activity assay system was prepared with 360 μM L-arginine solution and 0.2 mM NADPH [33], and then NOS enzymatic activity was measured according to the manufacturer's specification with Nitric Oxide Synthase Assay Kit (Beyotime, China).

2.6. Detection of NO production by fluorescence microscopy and flow cytometry

NO production was detected by NO-specific fluorescent probe 3-Amino, 4-aminomethyl-2′, 7′-difluoresceindiacetate (DAF-FM DA, Beyotime, China). MCF-7 cells were treated with or without 200 μM flavone for 72 h. After treatment, the cells were washed three times with PBS and then incubated with 5 μM DAF-FM DA for 30 min at 37 °C in dark. Then cells were washed three times with PBS and examined using fluorescence microscopy.

Flow cytometry assay was used to confirm our results. MCF-7 cells were treated with or without 200 μM flavone for 72 h. After treatment, the cells were washed three times with PBS and harvested with 0.25% EDTA-trypsin. Then, cells were resuspended in 5 μM DAF-FM DA and incubated at 37 °C in dark for 30 min. After incubation, cells were washed three times and resuspended in PBS. Then flow cytometry was performed to measure the fluorescence of NO, with excitation wavelength of 495 nm and emission wavelength of 515 nm.

2.7. Protein S-nitrosylation assay

MCF-7 cells were treated with or without 200 μM flavone for 72 h. Proteins were extracted according to the manufacturer's specification S-Nitrosylated Protein Detection Assay Kit (Cayman, USA) which is based on the “Biotin-switch” method. Using this method, protein free thiols were blocked with a blocking agent, and then any S-Nitrosothiols in samples were reduced to yield free thiol(s), and subsequently covalently labeled with maleimide–biotin. After the quantification of protein concentration, equal amounts proteins of control and flavone-treated cells were purified by streptavidin magnetic beads. After purification, streptavidin magnetic beads were washed by water (PH 6.7) for six

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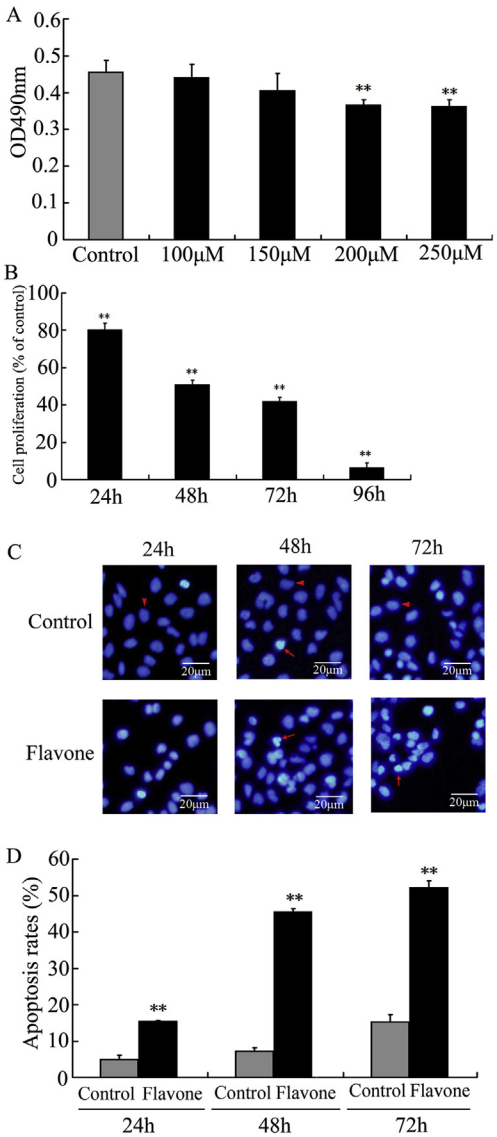

Fig. 1. Flavone inhibited cell viability and induced apoptosis in MCF-7 breast cancer cells with a concentration and time dependent manner. Cell viability was measured after treated by 0, 100, 150, 200 and 250 μM flavone for 24 h (A) and treated by 200 μM flavone for 24, 48, 72 and 96 h (B). (C) Representative photomicrographs of MCF-7 cells stained with Hoechst 33342 and PI fluorescent dye after incubated in absence (control) or presence of 200 μM flavone for 24, 48 and 72 h. The arrows showed the apoptotic cells distinguished by condensed or fragmented nuclei, and the arrowheads showed the live cells. (D) The quantitative results of apoptosis rates with different treatment time (**$P < 0.01$).

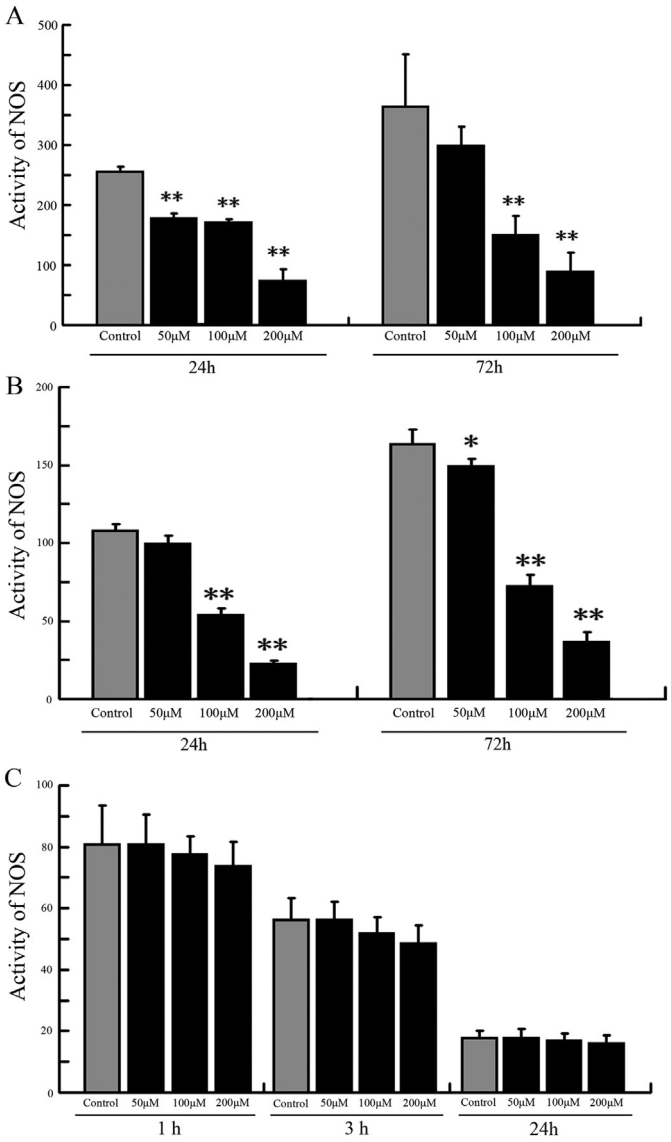

Fig. 2. Flavone inhibited NOS enzymatic activity. The activity of NOS was inhibited by 50, 100 and 200 μM flavone for 24 h and 72 h in MCF-7 cells (A) and MDA-MB-231 cells (B). NOS enzymatic activity in MCF-7 cell lysates was not affected by 50, 100 and 200 μM flavone after treated for 1, 3 and 24 h (C). NOS enzymatic activity was assayed by fluorescence microplate reader (*$P < 0.05$, **$P < 0.01$).

times, and then S-nitrosylated proteins were separated from streptavidin magnetic beads by 1:5 loading buffer. The S-nitrosylated proteins were detected by western blot.

2.8. Statistical analysis

All of the assays were performed three times independently at least. Data are presented as mean ± standard deviation. Statistical significance was assessed by Student's *t*-test. *$P < 0.05$, **$P < 0.01$.

3. Results

3.1. Flavone inhibited cell viability and induced apoptosis

After treated with 100, 150, 200 and 250 μM flavone for 24 h, the inhibition rates on cell viability were 3.26 ± 8.20%, 11.04 ± 10.45%, 19.64 ± 3.52% and 20.41 ± 4.15%. Cell viability was significantly inhibited by 200 and 250 μM flavone ($P < 0.01$), and without significant effects by 100 and 150 μM flavone (Fig. 1A). After treated with 200 μM flavone for 48, 72 and 96 h, the inhibitory effects on cell viability increased with the extension of treatment time (Fig. 1B). Hoechst and propidium iodide nuclear staining assay was used to detect flavone-induced apoptosis. Apoptosis rates of flavone-treated cells were higher than that of the controls. After treated by 200 μM flavone for 24, 48 and 72 h, the percentage of apoptotic cells was 15.15 ± 0.20%, 45.45 ± 1.00%, and 52.45 ± 1.03%, compared with 4.90 ± 1.23%, 7.15 ± 1.03% and 15.30 ± 6.58% in control groups (Fig. 1C and D). The percentage of necrotic cells was 2.49 ± 0.44%, 2.68 ± 1.66%, and 3.61 ± 0.63% after treated by 200 μM flavone for 24, 48 and 72 h, while the percentage of necrotic cells was 2.36 ± 0.39%, 2.20 ± 0.45% and 1.82 ± 0.91% in control groups. No matter treated for 24, 48 or 72 h, 200 μM flavone had no significant effect ($P > 0.05$) on cell necrosis. So the results of cell necrosis are not shown in the figure.

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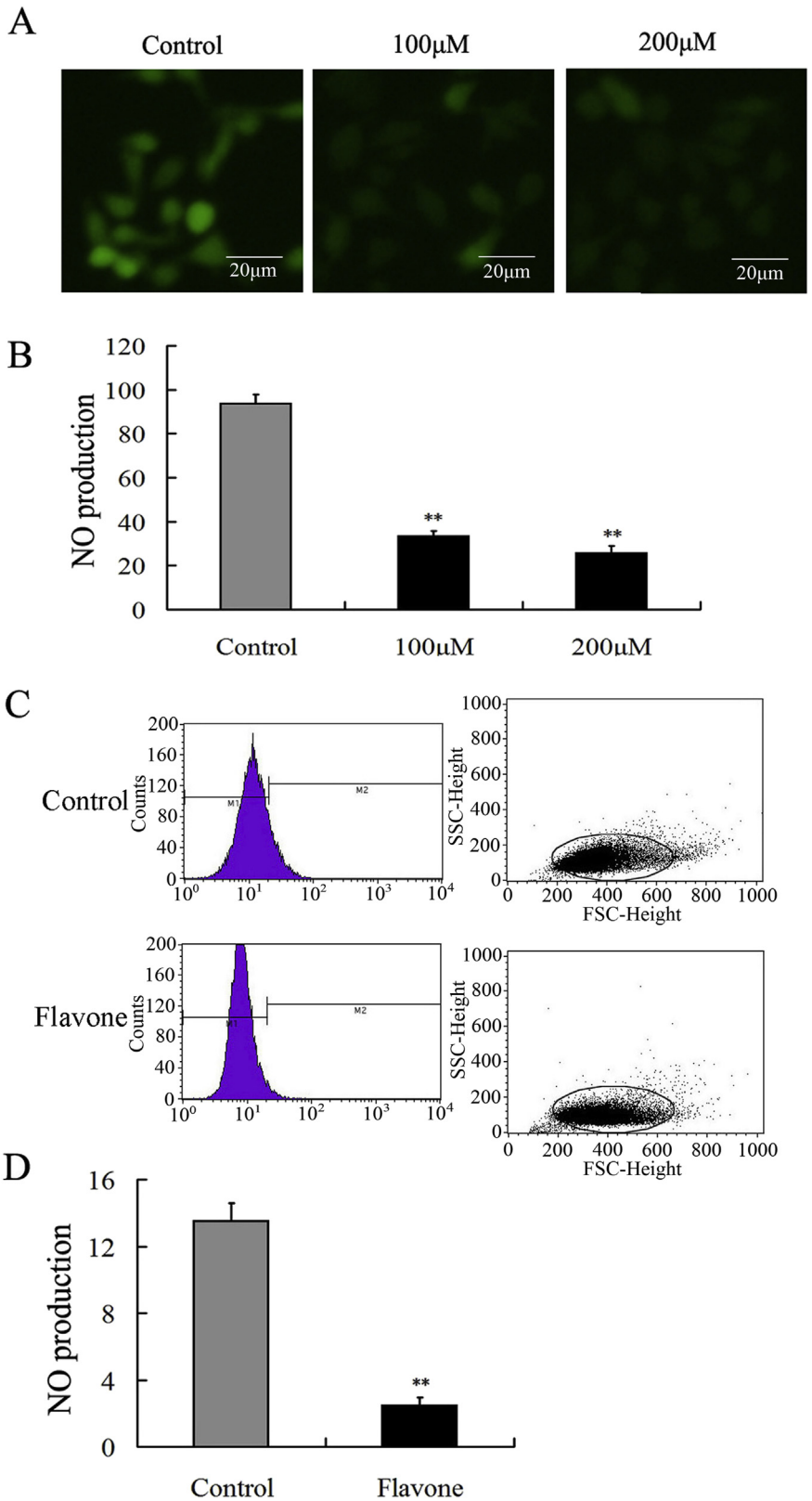

Fig. 3. Flavone decreased NO production in MCF-7 cells. Representative photomicrographs (A) and quantitative assays (B) of NO production in MCF-7 cells stained by DAF FM-DA fluorescent probe after incubated in absence (control) or presence of 100 and 200 μM flavone for 72 h. Results of flow cytometry (C) and quantitative results (D) of NO production in MCF-7 cells stained DAF FM-DA after treated by 200 μM flavone for 72 h.

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3.2. Flavone inhibited the activity of nitric oxide synthase (NOS)

The enzymatic activity of NOS was detected after MCF-7 and MDA-MB-231 cells were treated by 50, 100 and 200 μM flavone for 24 and 72 h. The results showed that, 50, 100 and 200 μM flavone all inhibited the activity of NOS. The inhibition rates were 30.4 ± 3.1%, 32.0 ± 2.1%, 70.5 ± 7.8% after 24 h treatment, and 18.03 ± 8.9%, 58.8 ± 8.8%, 76.0 ± 9.0% after 72 h treatment in MCF-7 cells (Fig. 2A), and 7.39 ± 4.73%, 49.88 ± 3.53%, 79.31 ± 2.32% for 24 h, and 8.93 ± 2.92%, 55.96 ± 4.48%, 77.63 ± 3.90% for 72 h treatment in MDA-MB-231 cells (Fig. 2B). But flavone exhibited no effects on the NOS activity in MCF-7 cell lysates after treatment for 1, 3 and 24 h (Fig. 2C).

3.3. Flavone inhibited the production of nitric oxide (NO)

Using NO-sensitive fluorescence probe DAF FM-DA, flavone-induced effect on NO generation was detected by fluorescence microscopy. The intensity of bright green fluorescence indicated the level of NO. The results showed that, after treated for 72 h, both 100 and 200 μM flavone inhibited the production of NO in MCF-7 cells significantly (Fig. 3A and B), inhibition rates were 64.5 ± 2.7% and 72.6 ± 3.4% respectively. The production of NO was also confirmed by flow cytometry and the inhibition rate is 81.7 ± 3.6% when MCF-7 cells were treated by 200 μM flavone for 72 h (Fig. 3C and D).

3.4. Flavone down-regulated S-nitrosylation of protein

Since flavone inhibited NO production in above results, the next assay detected the effect of flavone on S-nitrosylation of proteins, which is a post-translational modification caused by NO. MCF-7 cells were treated by 200 μM flavone for 72 h, and then proteins were extracted from control and flavone-treated MCF-7 cells by using “S-Nitrosylated Protein Detection Assay Kit”. All S-Nitrosylated proteins were labeled with maleimide–biotin in the process of protein extracting. After quantified the proteins of control and flavone-treated cells, we detected the total proteins by Western blot (Fig. 4A). Then the S-Nitrosylated proteins were purified and separated from equal amounts total proteins of control and flavone-treated cells by streptavidin magnetic beads, and detected by Western blot (Fig. 4B). Results showed that, S-nitrosylation of proteins was significantly down-regulated after treated by 200 μM flavone for 72 h in MCF-7 cells.

4. Discussion

As the basic structure of flavonoids, flavone has been shown to inhibit cell growth by inducing DNA fragmentation, cell cycle arrests and caspase-3 activation in human colon and breast cancer cell lines [27,29,30]. Furthermore, flavone has selectivity to cause cell death in cancer cells but not in nontumorigenic epithelial cells [27,28,34]. In this study, our results indicated flavone inhibited cell proliferation and induced apoptosis with a concentration- and time-dependent manner in the breast cancer cells (Fig. 1).

Some kinds of flavonoids have been shown to reduce NO production, such as Ampelopsin, Quercetin, Epigallocatechin-3-gallate, Puerarin and so on [20,21,25,26,35]. The flavone-induced cell apoptosis, activation of caspase-3-like and release of cytochrome C are all restrained by SNP in HT-29 colon cancer cells, suggesting that flavone's apoptosis-inducing function is related to NO [30]. Therefore, the flavone-induced effect on NO production was detected. The results by fluorescence microscopy and flow cytometry both indicated that flavone inhibited NO production in MCF-7 cells (Fig. 3). Interestingly, Wenzel et al. treated HT-29 cells for 3 h with SNP and flavone did not change NO production [30]. Flavone's NO inhibitory effect might have not performed with only 3 h treatment. This also implies that flavone regulates NO production by intervening synthesis instead of acting as a directly scavenger. Our results showed that the activity of NOS was inhibited by flavone after treated for 24 and 72 h in MCF-7 and in MDA-MB-231 breast cancer cells (Fig. 2A and B). This indicated that flavone decreases NO production by inhibiting the activity of NOS.

The enzymatic activity of NO synthase is highly associated with organelle. Endothelial NOS (eNOS) is predominantly membrane-bound and primarily located in Golgi apparatus and membranes of some vesicles. Inducible NOS (iNOS) is more active in cis-Golgi. The cytosol-located NOS are unstable in vitro in a purified form. Furthermore, NF-κB, the upstream regulator of NOS, is located in endoplasmic reticulum [36–40]. Our results showed that, flavone did not affect NOS activity in the lysate of MCF-7 cells after 1, 3 and 24 h treatment (Fig. 2C), this indicated that flavone didn't affect NOS activity directly, and the flavone-induced inhibitory effect on NOS enzymatic activity is dependent on intact subcellular structures.

Nitric oxide plays its role partly by mediating S-nitrosylation of cysteine residues in cellular signal transduction [41]. After we determined flavone inhibits NO production, we further detected flavone-induced regulation on protein S-nitrosylation. Our results showed that, after treated by 200 μM flavone for 72 h, the total protein S-nitrosylation was decreased in MCF-7 cells (Fig. 4). S-nitrosylation occurs on lots of cancer-related proteins including p53, caspase, MKPs, Bcl-2, Fas, C-Src tyrosine kinase and actin, which regulate multiple cellular processes involving proliferation, apoptosis, angiogenesis, migration, invasion and metastasis [12,42]. S-nitrosoglutathione reductase (GSNOR) is a pivotal protein which degrades the main non-protein S-nitrosothiol (SNO). The livers of GSNOR-deficient mice exhibit significantly high levels of protein S-nitrosylation, accompanied with 10-fold higher incidence of spontaneous hepatocellular carcinoma than controls [43]. Furthermore, the susceptibility to *Klebsiella* pneumonia of GSNOR-deficient mice is increased [44].

In conclusion, in this study, we found that flavone inhibits cell proliferation, induces apoptosis, reduces the nitric oxide production by inhibiting activity of NOS and then down-regulates protein S-nitrosylation in breast cancer cells. There are no reports about flavone-induced effects on S-nitrosylation until now. Our study indicates a novel mechanism, NOS/NO/S-nitrosylation, for flavone's

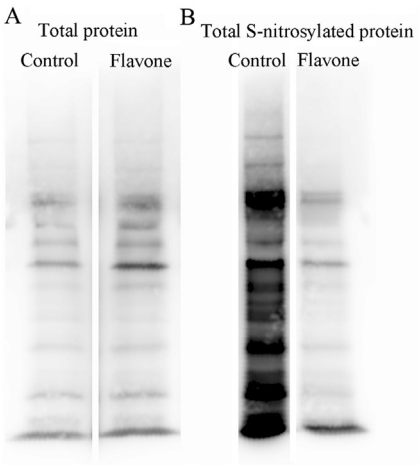

Fig. 4. Flavone decreased protein S-nitrosylation in MCF-7 cells. Total protein (A) and total S-nitrosylated protein (B) were shown by western blot in absence (control) or presence of 200 μM flavone for 72 h.

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anti-cancer function, and also provides experimental evidence for the research of other flavonoids on nitric oxide and S-nitrosylation.

Conflict of interest

All authors declare no conflict of interest.

Acknowledgments

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