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Modulation of FoxO1 phosphorylation/acetylation by baicalin during aging

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Abstract

Baicalin is a flavonoid known to modify various redox-related biological activities. Included is its ability to suppress reactive species (RS) producing activity and modulate nuclear factor-κB through cellular redox regulation with enhanced thiol ability.

FoxO regulates various genes that are known to be involved in cellular metabolism related to cell death and the oxidative stress response. One such case is the prevention of FoxO1 expression by activated insulin-induced phosphatidylinositol 3-kinase (PI3K)/Akt, which leads to increased oxidative stress and aging processes.

In the present study, we attempted to elucidate the molecular modulation of antioxidant baicalin on the insulin-induced FoxO1 inactivation. We used HEK293T cultured cells and kidney tissue isolated from 24-month-old rats treated with baicalin at a dose of 10 or 20 mg/kg/day for 10 days.

We found that baicalin enhanced catalase and suppressed RS production in cell system and in isolated kidney tissue in contrast to the nontreated aged rats. Results also showed activation of insulin signaling (PI3K/Akt), FoxO1 phosphorylation/acetylation and the down-regulation of catalase and manganese superoxide dismutase, both of which are FoxO1-targeting genes. Furthermore, baicalin-treated rats showed a decreased FoxO1 phosphorylation via PI3K/Akt cascade and FoxO1 acetylation by the cAMP-response element-binding protein binding protein (CBP). These results strongly suggest that treatment with baicalin influenced phosphorylation/acetylation of FoxO1 by up-regulating PI3K/Akt signaling through insulin in aged rats. Our results further reveal that baicalin regulated FoxO1 phosphorylation via PI3K/Akt by insulin and FoxO1 acetylation by the interaction of CBP and SIRT1, leading to changes in catalase gene expression during aging.

Keywords: Acetylation; Aging; Baicalin; Foxo1; Insulin; Phosphorylation; Reactive species

1. Introduction

Flavonoids are polyphenolic compounds found ubiquitously in food plants and vegetables. Many flavonoids possess beneficial properties such as antitumor, antiplatelet, anti-ischemic and anti-inflammatory properties [1]. These effects are believed to result mainly from their potent antioxidative properties [2]. Among these are the antioxidative action of baicalin to neutralize reactive species (RS) [3], pro-matrix metalloproteinase, proinflammatory cytokines and prostaglandin E2 in leukocytes, and microglial and neuron cells [4]. Recently, our laboratory reported [5] on baicalin as an efficient RS scavenger and modulator of proinflammatory nuclear factor (NF)-κB (and its gene expression) through the MAPK pathway, making it a potentially useful antioxidant and anti-inflammatory agent. Therefore, we search to find the effect of baicalin in association with forkhead box O transcription factor (FoxO1) and SIRT1, both of which are shown to regulate aging in yeast and invertebrate models under conditions of increased oxidative stress [6,7].

The evolutionally conserved FoxO family of transcription factors consists of FoxO1, FoxO3a, FoxO4 and FoxO6 in mammals [8]. A recent series of investigations have demonstrated that FoxO factors play key roles in inducing various downstream target genes that regulate cellular metabolism involving cell cycle, cell death and the oxidative stress response [8,9]. One of the key regulatory mechanisms of FoxO factors involves the phosphorylation reaction. Phosphorylated FoxO by protein kinase B in response to insulin or several growth factors (PKB, also known as Akt) is allowed to translocate from the nucleus to the cytoplasm [9-11]. Another regulatory step involves the acetylation of FoxO as a posttranslational modification [12,13]. cAMP-response element-binding protein (CREB)-binding protein (CBP) triggers the transactivation function of both FoxO1 and FoxO4, whereas following the acetylation of these FoxOs by CBP leads to the attenuation of their transcriptional activity [12,13]. However, the acetylation of FoxO1 can be reversed by silent information regulator 2 (Sir2) through its NAD-dependent

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deacetylase activity, consequently activating transcription mediated by FoxO1 [13]. When Sir2 is inactive, acetylated FoxO1 by CBP dissociates from DNA, eliciting Akt signaling to phosphorylate FoxO1. Therefore, acetylated FoxO1 becomes more sensitive to Akt-dependent phosphorylation [14], and emerging evidence suggests that phosphatidylinositol 3-kinase (PI3K)/Akt signaling regulates cellular status of oxidative stress [15,16].

FoxO has been shown to reduce the level of cellular oxidative stress by directly increasing mRNA and protein levels of manganese superoxide dismutase (MnSOD) and catalase [17]. It has been reported that the beneficial effects of SIRT1 overexpression *in vivo* are in part an up-regulation of catalase that prevents oxidative-stress-induced cardiomyocyte damage. The up-regulation of catalase seems to occur from the transcriptional activation of FoxO1 because the overexpression of a dominant negative form of FoxO1 prevents the SIRT1-induced catalase expression. Indeed, SIRT1 is reported to stimulate catalase expression and resistance to oxidative stress via Foxo1 in the heart [18-20]. However, age-related FoxO1 phosphorylation/acetylation by baicalin has not been reported and at present is unknown.

In the present study, we investigated the effects of baicalin on phosphorylation and acetylation of FoxO1 through the PI3K/Akt pathway and RS-related antioxidant enzyme gene expression in 24-month-old rat kidney and the action mechanisms of baicalin in cultured HEK293T cells.

2. Materials and methods

2.1. Animals

Specific pathogen-free male Fischer 344 rats (6 and 24 months of age) were obtained from Samtako (Osan, Korea) and were treated a diet of the following composition: 14 g casein, 0.18 g L-cysteine, 46.57 g corn starch, 15.5 g dextrinized corn starch, 10 g sucrose, 5 g fiber, 4 g soybean oil, 3.5 g AIN-93 mineral mix, 1 g AIN-93 vitamin mix, 0.25 g choline bitartrate and 0.8 mg tert-butylhydroquinone (TBNQ).

Powdered chow was treated with baicalin and fed to the old (age 24 months) rats at a dose of either 10 or 20 mg/kg/day. After 10 days, the rats were killed.

The rats were sacrificed by decapitation, and the kidneys were quickly removed and rinsed in iced-cold buffer [100 mM Tris, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 1 μM pepstatin, 2 μM sodium orthovanadate (pH 7.4)]. The tissue was immediately frozen in liquid nitrogen and stored at −80°C. The kidney was used for the study because of its vulnerability to oxidative stress and the responsiveness to age-related changes, including dietary manipulation [19,20]. We are thankful to the Aging Tissue Bank funded by the National Research Foundation of Korea for supplying aged tissue.

2.2. Cell culture system

HEK293T cells (human embryo kidney 293T cells) were obtained from ATCC (American Type Culture Collection, Rockville, MD, USA). HEK293T cells were cultured in Dulbecco's modified Eagle media (DMEM) (Nissui Co., Tokyo, Japan) supplemented with 10% heat-inactivated (56°C for 30 min) fetal bovine serum (Gibco, Grand Island, NY, USA), 233.6 mg/ml glutamine, 72 μg/ml penicillin streptomycin and 0.25 μg/ml amphotericin B, and adjusted to pH 7.4–7.6 with $NaHCO_3$ in an atmosphere of 5% CO_2. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

2.3. Materials

Baicalin, insulin, and splitomicin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 2′,7′-Dichlorodihydrofluorescein diacetate (DCFDA) was obtained from Molecular Probes, Inc. (Eugene, OR, USA). LY294002 was obtained from Cell Signaling Technology, Inc. (MA, USA). The radionucleotide [γ-^{32}P]-ATP (250 μCi) and Western blotting detection reagents were obtained from Amersham (Bucks, UK). RNAzol B was obtained from TEL-TEST, Inc. (Friendwood, TX, USA). Antibodies against FoxO1, p-FoxO1 (Thr 24), catalase, MnSOD, β-actin, histone H1, PI3K (p85α), p-Akt, total-Akt, CBP and SIRT1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against p-FoxO1 (Ser 256) were obtained from Cell Signaling Technology, Inc. (MA, USA). Antibodies against acetyl-lysine were obtained from Upstate. Anti-rabbit IgG horseradish-peroxidase-conjugated antibody and anti-mouse IgG horseradish-peroxidase-conjugated antibody were obtained from Amersham Pharmacia Biotech (Bucks, UK). Horseradish-peroxidase-conjugated donkey anti-sheep/goat IgG was purchased from Serotec (Oxford, UK). Polyvinylidene difluoride (PVDF) membranes were obtained from Millipore Corporation (Bedford, MA, USA).

2.4. Nuclear extract preparation

The frozen rat kidney tissue (0.2–0.4 g) was rinsed in phosphate-buffered saline and then transferred to the Dounce tissue grinder (Wheaton Manufacturers, NJ, USA). Solution A [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF] was added at a ratio of 2.5 ml/g tissue. Five strokes of pestle were used to homogenize tissue to liquid mass. After the addition of NP-40 (0.5%), five additional strokes of homogenization were performed. The homogenates were transferred to Eppendorf tubes and centrifuged in a microcentrifuge (Beckman) for 1 min.

The supernatant contained mostly cytoplasmic constituents. To yield the nuclear pellet, 400 μl of solution C [20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM each of EDTA, EGTA, DTT and PMSF] was added, and the tubes were mixed thoroughly and placed on a small rotatory shaker for 15 min. Finally, the mixture was centrifuged at 12,000 rpm for 3 min in the microcentrifuge. The supernatant containing the proteins from the nuclear extract was removed and transferred carefully to a fresh tube. The nuclear extract was frozen at −80°C in aliquots until Western blotting was done. The protein content of each sample was determined using the bicinchoninic acid protein assay (Sigma).

2.5. Western blotting

Western blotting was carried out as described previously [21]. Homogenized samples were boiled for 5 min with a gel-loading buffer [125 mM Tris-Cl, 4% sodium dodecyl sulfate (SDS), 10% 2-mercaptoethanol (pH 6.8), 0.2% bromphenol blue] in ratio of 1:1. Total protein equivalents for each sample were separated by SDS–polyacrylamide gel electrophoresis (PAGE) using acrylamide gels as described by Laemmli [22], and transferred to PVDF membrane at 15 V for 1 h in a semidry transfer system. The membrane was immediately placed into a blocking buffer (1% nonfat milk) in 10 mM Tris (pH 7.5), 100 mM NaCl and 0.1% Tween-20. The blot was allowed to block at room temperature for 1 h. The membrane was incubated with specific primary antibody at 25°C for 1 h, followed by a horseradish-peroxidase-conjugated secondary antibody at 25°C for 1 h. Antibody labeling was detected using enhanced chemiluminescence per the manufacturer's instructions. Prestained protein markers were used for molecular weight determinations.

2.6. Immunoprecipitation of nuclear extracts

Nuclear extracts were subjected to immunoprecipitation (IP) [23] in a buffer containing 40 mM Tris-HCl (pH 7.6), 120 mM NaCl, 20 mM β-glycerophosphate, 20 mM NaF, 2 mM sodium orthovanadate, 5 mM EDTA, 1 mM PMSF, 0.1% Nonidet P40 (NP40) with leupeptin (2 μg/ml), aprotinin (1 μ/ml) and pepstatin A (1 μ/ml). One thousand micrograms of nuclear extracts was incubated with 50% slurry of protein A agarose for 30 min at 4°C for preclearing. After incubation, nuclear extracts were centrifuged at 12,000*g* at 4°C for 15 min. The nuclear extracts were then incubated overnight with the respective antibody at 4°C followed by incubation overnight at 4°C with 50% slurry of protein A agarose. After washing of the immunoprecipitates three times with IP buffer, the immunoprecipitated proteins were analyzed by SDS-PAGE followed by Western blotting analysis as described previously.

2.7. Insulin assay

Insulin level was determined using the rat insulin enzyme-linked immunosorbent assay kit (Shibayagi Co., Japan). One hundred microliters of biotin-conjugated anti-insulin solution was added to 10 μl of sample in each well. Chromogenic substrate solution was added to wells after addition of HRP-conjugated avidin solution to each well. After 30-min incubation at room temperature, 100 μl of reaction stopper was added. The optical density was measured at 450 nm using a microplate reader (TECAN, Salzburg, Austria).

2.8. Electrophoretic mobility shift (EMSA) assay

The EMSA method was used to characterize the binding activities of FoxO in nuclear extracts [24]. The FoxO oligonucleotide was 5′-TTA GTC ATT TTG TTT GTT CAT A-3′. Protein–DNA binding assays were performed with 10 μg of nuclear protein. To minimize salt on binding, the concentration of salt was adjusted to the same level in all samples. Unspecific binding was blocked by using 1 μg of poly(dI-dC)·poly(dI-dC). The binding medium contains 5% glycerol, 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM EDTA, 2 mM DTT 1% NP40 and 10 mM Tris/HCl (pH 7.5). In each reaction, 20,000 cpm of radiolabeled probe was included. Samples were incubated at room temperature for 20 min, and the nuclear protein-^{32}P-labeled oligonucleotide complex was separated from free ^{32}P-labeled oligonucleotide by electrophoresis through a 5% native polyacrylamide gel in a running buffer containing 50 mM Tris (pH 8.0), 45 mM borate and 0.5 mM EDTA. After separation was achieved, the gel was vacuum dried for autoradiography and exposed to Fuji X-ray film for 1–2 days at −80°C.

Table 1
Change of body weight and food intake

	Young	Old	Old+Bai 10 mg/kg	Old+Bai 20 mg/kg
Initial body weight (g)	355.52±21.16	428.55±39.72	427.28±38.42	423.29±32.89
Final body weight (g)	415.46±30.87	474.26±21.59†	429.56±29.71	430.12±27.62*
Food intake (g/day)	23.32±3.16	31.12±4.98	29.42±5.26	27.33±4.12

Bai: baicalin.
* $P<.05$ vs. age-matched rats.
† $P<.05$ vs. young group of rats.

2.9. RS measurement

For the determination of intracellular RS generation, HEK293T cells were seeded in a 96-well plate. After 1 day, the medium was changed to a fresh serum-free medium. The cells were treated with or without baicalin and LY294002 and were preincubated for 1 h. After treatment with insulin (100 nM) for 30 min, the medium was replaced with fresh serum-free medium, and DCFDA (fc 2.5 μM) was added. The fluorescence intensity of DCF was measured every 5 min for 30 min using the microplate fluorescence reader TECAN (Salzburg, Austria) with excitation and emission wavelengths of 485 and 535 nm, respectively.

2.10. Statistical analysis

Data are expressed as mean±standard error (S.E.). Results were analyzed statistically by one-way analysis of variance (ANOVA) followed by the Fisher's protected LSD post hoc test. Values of $P<.05$ were considered statistically significant.

3. Results

3.1. Effects of baicalin on FoxO1 phosphorylation and acetylation in aged rats

Transcriptional activity of FoxO family proteins has been reported to be increased by the reduction of insulin [10]. To verify whether age and baicalin affect FoxO1 modification, phosphorylation and acetylation were examined in aged rat kidneys.

Data on body weight and food intake are shown in Table 1. The food intakes of rats in both groups were similar, but interestingly, body weight was suppressed by high-dose baicalin (Table 1).

FoxO1 phosphorylation showed higher levels in old rats compared to young rats. The baicalin-treated group showed lower FoxO levels compared to the non-baicalin-treated control group of the same age (Fig. 1A). To determine the extent of age-related activation of FoxO, EMSA was carried out with nuclear proteins. Results showed age-induced decreases in the nuclear binding activity of FoxO in the control group, while little change was detected in baicalin-treated rats, even in the senescent, 24-month-old rat kidney (Fig. 1B). Binding specificity of the FoxO complex was demonstrated using a 100-fold excess of an unlabeled oligonucleotide, which competed for binding (Fig. 1B, lane 6).

As shown in Fig. 1C, acetylase CBP, as a coactivator, was enhanced with age and led to FoxO1 acetylation, which was decreased by baicalin treatment. On the other hand, deacetylase SIRT1 decreased with age, but was increased by baicalin treatment.

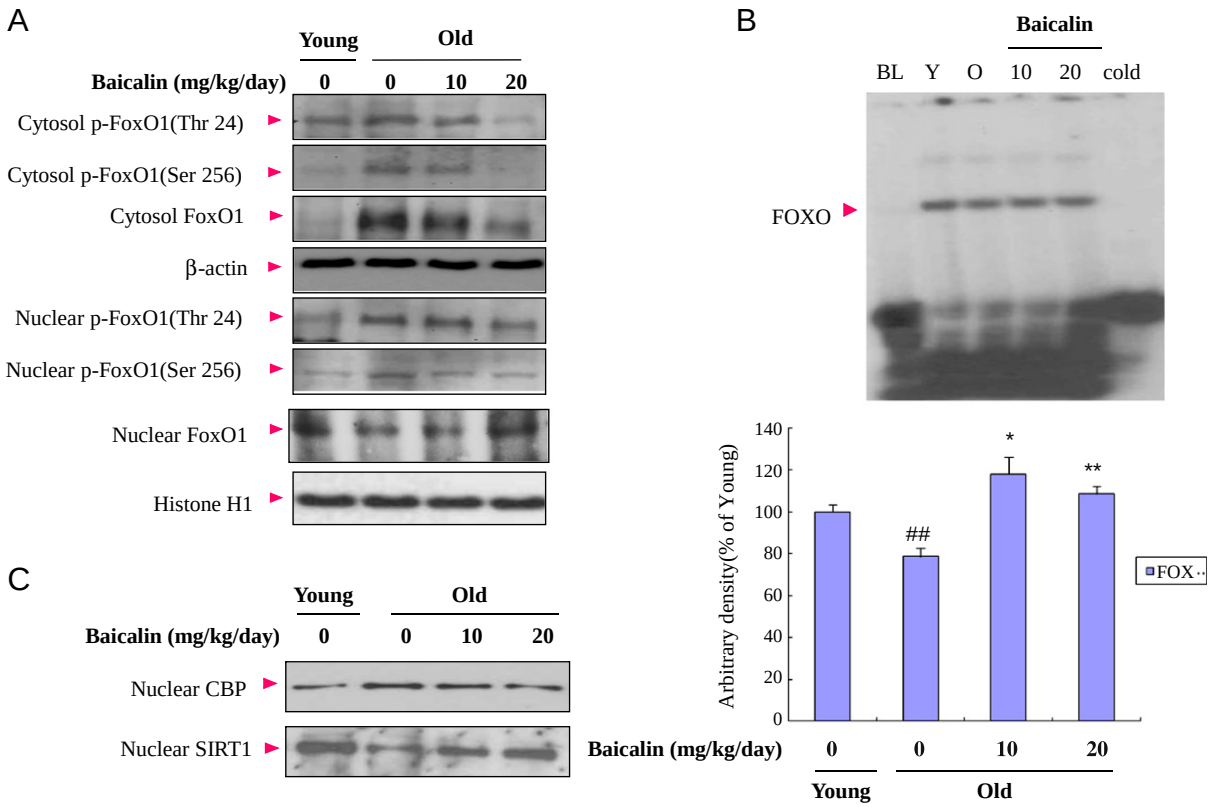

Fig. 1. Effects of age and baicalin on FoxO1 phosphorylation and acetylation. Increased FoxO1 phosphorylation/acetylation during aging and its modulation by baicalin were shown. Western blotting analysis of renal nuclear and cytosol (A) FoxO1 was performed from nuclear and cytosol protein of non-baicalin-treated and baicalin-treated rats. A representative result is presented from three experiments. (B) Baicalin increased age-related suppression of FoxO binding activity. The EMSA method was used to compare nuclear FoxO binding activities in kidney nuclear protein from non-baicalin-treated and baicalin-treated rats. Lane 1: probe without nuclear protein sample (BL); lanes 2–5: kidney nuclear protein samples of non-baicalin-treated and baicalin-treated rats aged 6 and 24 months; lane 6: competition assay using 100-fold excess of unlabeled Forkhead responsive element (FHRE) oligonucleotide. A representative result is presented from three experiments. (C) Effect of age and baicalin on CBP and SIRT1 expression. Western blotting analysis was performed to detect CBP and SIRT1 protein levels from non-baicalin-treated and baicalin-treated rats at ages 6 and 24 months. Results of one-factor ANOVA: ##$P<.01$ vs. young group of rats; *$P<.05$ vs. age-matched rats; **$P<.01$ vs. age-matched rats.

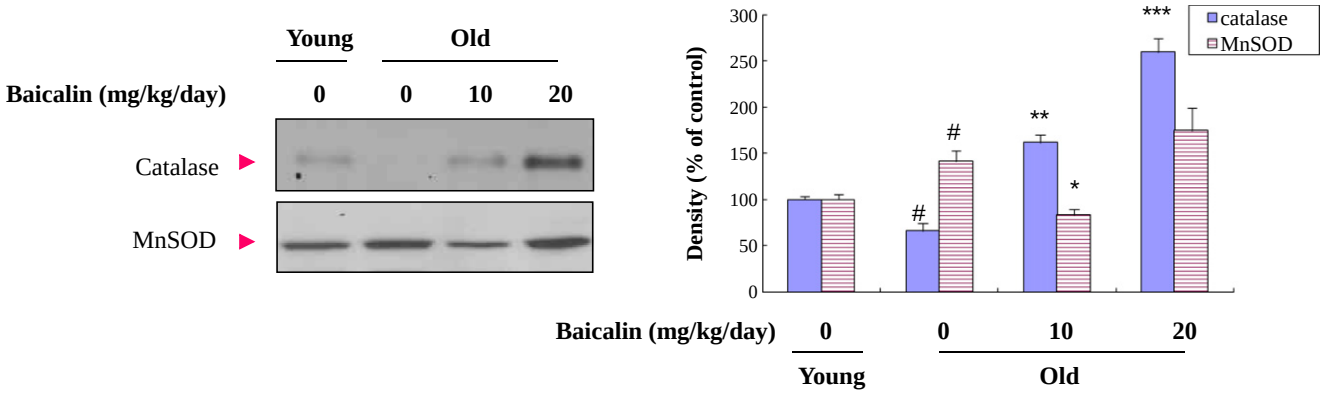

Fig. 2. Effects of age and baicalin on gene expression of FoxO1-dependent genes, MnSOD and catalase. Western blotting analysis was performed to detect catalase and MnSOD protein levels from non-baicalin-treated and baicalin-treated rats at ages 6 and 24 months. One representative blot of each protein is shown from three experiments that yielded similar results, respectively. Results of one-factor ANOVA: $^{\#}P<.05$ vs. young group of rats; $^{*}P<.05$ vs. age-matched rats; $^{**}P<.01$ vs. age-matched rats; $^{***}P<.001$ vs. age-matched rats.

These data indicate that baicalin raises DNA binding activity by decreasing FoxO1 phosphorylation and inhibiting FoxO1 acetylation.

3.2. Effects of baicalin on gene expression of FoxO1-dependent target genes, MnSOD and catalase

MnSOD and catalase are two major antioxidant enzymes that play a role in the protection against oxidative stress through the metabolism of RS. Protein levels of antioxidant enzymes with age were determined by Western blotting. As shown in Fig. 2, MnSOD levels were unchanged during aging, but catalase levels decreased with age. On the other hand, baicalin treatment was shown to increase protein level of catalase compared to the nontreated group.

3.3. Modulation of RS by baicalin

To assess the overall age-related oxidative status and its modulation by baicalin, total RS was measured with DCFDA probe in kidney homogenates. The results showed an increased RS level with age that was significantly suppressed by treatment with high-dose baicalin (Fig. 3). These results indicated that, during the aging process, baicalin aids to suppress RS through the up-regulation of MnSOD and catalase by modulating FoxO1 dephosphorylation and deacetylation.

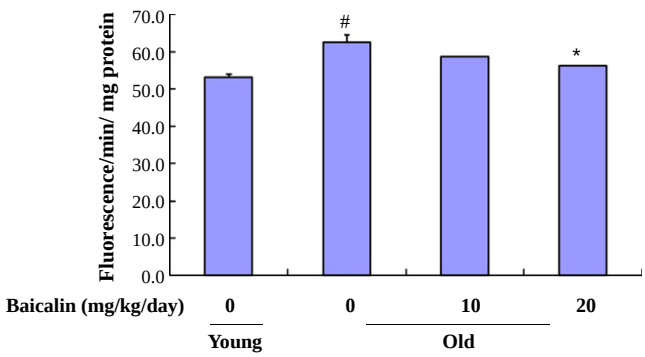

Fig. 3. Modulation of RS by baicalin. RS generation in aged rat was determined, observing the age/baicalin effect using the DCFHDA method in kidney homogenate. Each value is the mean±S.E. of five rats. Young, 6-month-old baicalin-untreated rat; old, 24-month-old baicalin-untreated rat. Each value is the mean±S.E. of five rats. Results of one-factor ANOVA: $^{\#}P<.05$ vs. young group of rats; $^{*}P<.05$ vs. age-matched rats.

3.4. Effects of baicalin on insulin level and its signaling molecules

Binding of insulin and growth factors to specific receptor tyrosine kinases activates PI3K and the serine-threonine kinase Akt. Akt promotes cell survival and proliferation in part by directly phosphorylating and inhibiting members of the FoxO subfamily of forkhead transcription factors [10,11,25].

We therefore examined the signaling molecules that lead to Akt activation and insulin levels with age. Insulin levels were shown to increase with age, while baicalin treatment markedly reduced insulin levels with age (Fig. 4A). Insulin expresses its action by activating the PI3K and its downstream target Akt. Therefore, to determine whether the change in the FoxO phosphorylation is caused by activation of the PI3K/Akt pathway, phosphorylated Akt (the active form of Akt) was investigated. Although total Akt amounts did not change, phosphorylated Akt in Ser473 was shown to increase with age (Fig. 4B). Conversely, baicalin treatment was observed to suppress the age-related increase of phosphorylated Akt (Fig. 4B).

These data suggest that PI3K/Akt signaling up-regulated by increased insulin levels might be associated with FoxO phosphorylation during the aging process and that baicalin treatment reversed these phenomena.

3.5. FoxO1-interaction coactivator CBP and its modulation by baicalin

Several papers have reported reversible acetylation of FoxO by CBP/p300 in mammalian cell lines [6,13]. Moreover, hydrogen peroxide stress is reported to result in an increased binding of FoxO to CBP/p300 that leads to increased FoxO acetylation [6]. In our current IP study, an interaction between FoxO1 and CBP during aging was demonstrated. As shown in Fig. 5A and B, the interaction between CBP and FoxO1 increased during aging, but was modulated by baicalin treatment. These results suggest that the aging process may induce the acetylation of FoxO1 by CBP and be reversed by baicalin.

3.6. Regulation of FoxO1 phosphorylation through PI3K/Akt pathway and acetylation by insulin and baicalin

To confirm whether age and baicalin affect FoxO1 phosphorylation and acetylation, we examined the effect of baicalin on FoxO phosphorylation and acetylation. For these experiments, serum-starved HEK293T cells were treated with 40 μM baicalin for 1 h prior to a 30-min treatment with 100 nM insulin. As shown in Fig. 6A, insulin enhanced the phosphorylation of FoxO1 on Ser 256 and Thr 24, which were targeted by baicalin. We observed the induction of

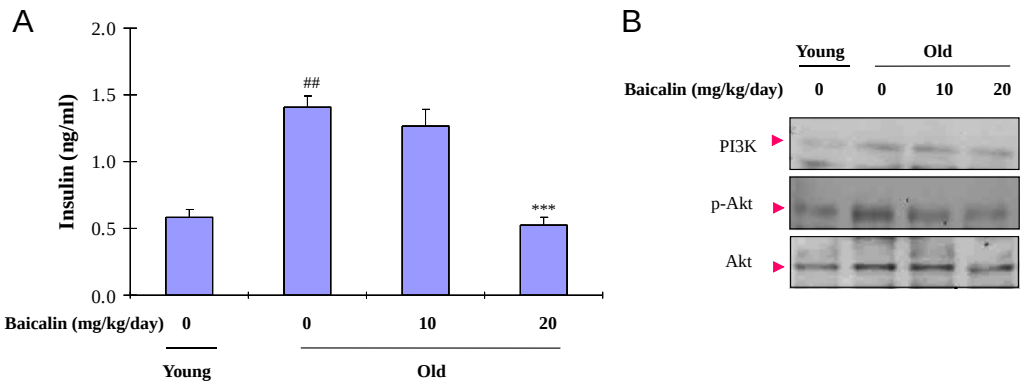

Fig. 4. Effects of age and baicalin on insulin level and insulin signaling molecules. (A) Levels of insulin in young and old, non-baicalin-treated and baicalin-treated rat serum were measured by reagent kit Shibayagi insulin (n=6 in each group). (B) Baicalin prevented age-related activation of the PI3K/Akt pathway. Western blotting analysis was performed to detect PI3K, total and phosphorylated Akt (Ser 473) cytoplasmic extracts (60 μg protein) from non-baicalin-treated or baicalin-treated rats aged 6 and 24 months old. One representative result of each protein is shown from three experiments that yielded similar results, respectively. Results of one-factor ANOVA: $^{\#\#}P<.01$ vs. young group of rats; $^{***}P<.001$ vs. age-matched rats.

FoxO1 acetylation by insulin and investigated the ability to block induction of FoxO1 acetylation by baicalin. The level of SIRT1 was reduced in insulin-induced HEK293T cells by baicalin (Fig. 6A).

We examined the effects of baicalin and PI3K/Akt inhibitor, LY294002 on FoxO phosphorylation and SIRT1 inhibitor, splitomicin on FoxO acetylation. In these experiments, serum-starved HEK293T cells were treated with LY294002, splitomicin and baicalin for 1 h prior to a 30-min treatment with 100 nM insulin. The FoxO is negatively regulated by phosphorylation through the PI3K/Akt pathway [8]. Because we have identified insulin-induced acetylation as an additional modification for FoxO factors, we next tried to elucidate the relation between acetylation and phosphorylation of FoxO1. As shown in Fig. 6B, insulin enhanced CBP and phosphorylation of FoxO1 on Ser 256 and Thr 24, which were targeted by LY294002 and baicalin. Moreover, the level of SIRT1 was reduced in insulin-induced HEK293T cells by LY294002 and baicalin. On the other hand, the phosphorylation level of FoxO1 at Thr 24 and Ser 256 was increased when cells were incubated with deacetylase inhibitor (Fig. 6B), suggesting that insulin may induce acetylation of FoxO1 by CBP. Taken together, these findings provide evidence that baicalin regulates the acetylation and phosphorylation of FoxO1 during aging.

3.7. Baicalin modulates insulin-induced oxidative stress in cultured cells

Evidence indicates that the mammalian FoxO up-regulates the radical scavenger genes, catalases that have a protective effect against oxidative damage in human cells [26,27]. Results show that insulin-induced HEK293T cells exhibited an increase of RS and that baicalin treatment decreased RS (Fig. 7) consistent with the Akt inhibitor LY294002. These results suggest that baicalin may modulate the redox status and catalase expression by regulating PI3K/Akt-FoxO1 signaling.

4. Discussion

Initial clues that PI3K controls FoxO activity came from studies performed with the nematode *Caenorhabditis elegans* [28]. For example, genetic studies established that PI3K suppresses the function of DAF-16, a forkhead transcription factor [28] that later was found to be critical for proper control of metabolism and cell survival [29]. At molecular levels, FoxO proteins bind to insulin response element in the proximal promoter [30,31] of target genes involved in cell survival, cell cycles, DNA repair and insulin sensitivity [12,31] and stimulate their transcription.

Several studies have also reported the modulation of FoxO transcription factors by many natural products through insulin signaling. For example, kaempferol caused a translocation of the *C. elegans* FoxO1 factor [32], and epigallocatechin gallate (EGCG) prevented the development of cardiac hypertrophy through RS-dependent and -independent mechanisms [33]. Further, the anti-angiogenic effects of EGCG were through the activation of FoxO by inhibiting PI3K/Akt [34]. However, to date, the putative action of baicalin on the modulation of FoxO at the molecular level has not been fully elucidated. Recent data suggest that FoxO can be activated by resveratrol, resulting in the up-regulation of genes

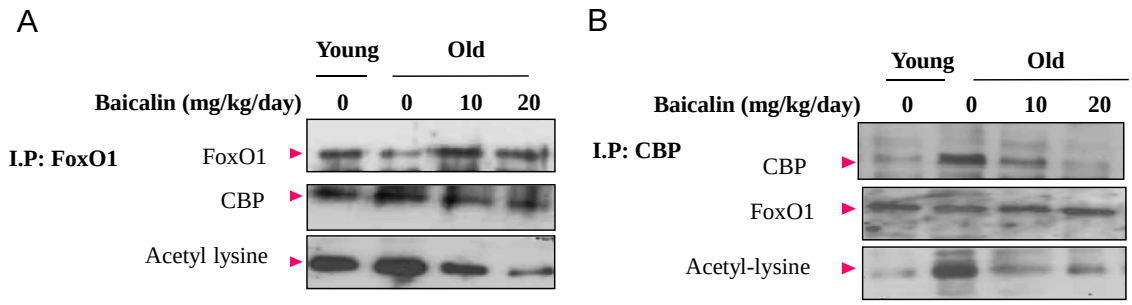

Fig. 5. FoxO1 interaction coactivator CBP and its modulation by baicalin. Nuclear extracts were prepared from young and old rat kidneys. Immunoprecipitated FoxO1 was determined to be physically associated with CBP by Western blotting.

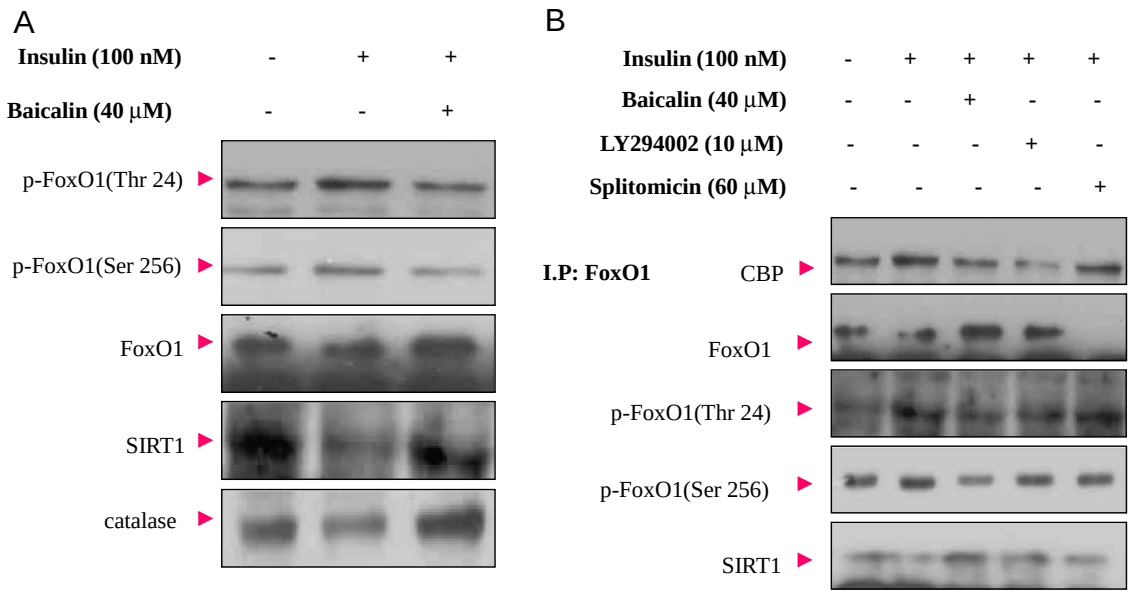

Fig. 6. Regulation of FoxO phosphorylation through PI3K/Akt pathway and acetylation. HEK293T cells were grown to confluence of 80% in 100-mm dishes in the DMEM medium. Cells were pretreated (1 h) with baicalin and then stimulated with 100 nM insulin. (A) After stimulation with insulin in the absence (−) or presence (+) of baicalin 40 μM, FoxO1 phosphorylation and deacetylation were determined from the cell extract. (B) After stimulation with insulin in the absence (−) or presence (+) of LY294002 (10 μM), baicalin and deacetylase inhibitor (splitomicin, 60 μM), the cells lysed were immunoprecipitated with anti-FoxO1 antibody, followed by Western blotting.

involved in the antioxidant pathway and activation of NF-κB, which typically up-regulates the expression of prosurvival genes such as MnSOD [35].

Recent work on baicalin brought out several interesting new findings more relevant to our current study. Yuling et al. [36] reported that mice fed a baicalin-containing diet for 50 days showed an increased antioxidative status and decreased iron content and lipid peroxidation in liver after iron overload. Also, baicalin treatment suppressed systemic inflammatory stress by reducing serum tumor necrosis factor α levels and by a significant reduction in serum insulin and glucose levels in ameliorated insulin resistance [37]. In our recent study, we observed that the short-term feeding of baicalin modulated age-related NF-κB activity in rat [5].

In the present study, it was found that the down-regulation of catalase, an antioxidant enzyme, through FoxO1 phosphorylation and acetylation occurred during aging, which was effectively prevented by short-term baicalin treatment. This new finding may provide evidence of baicalin's link to insulin signaling and oxidative stress during aging. This study also showed that serum insulin levels increase with age and that baicalin-treated rats showed suppressed increases in these levels (Fig. 4A). Insulin expresses its action by activating the PI3K and its downstream target Akt. Changes in the insulin level and its modulation are deemed to be important because the decreased FoxO binding activity (Fig. 1B) that results during aging is blunted by activated PI3K/Akt, which is insulin dependent. FoxO1 phosphorylation showed higher levels in old rats compared to young rats. The rats in the baicalin-treated group were shown to have lower FoxO1 levels when compared with the baicalin-treated control group of the same age (Fig. 1A). Also, CBP was enhanced by age, leading to FoxO1 acetylation, but decreased by baicalin. On the other hand, deacetylase SIRT1 was decreased by age and increased by baicalin treatment. These data indicate that baicalin raised DNA binding activity through decreased FoxO1 phosphorylation and inhibited the acetylation of FoxO1.

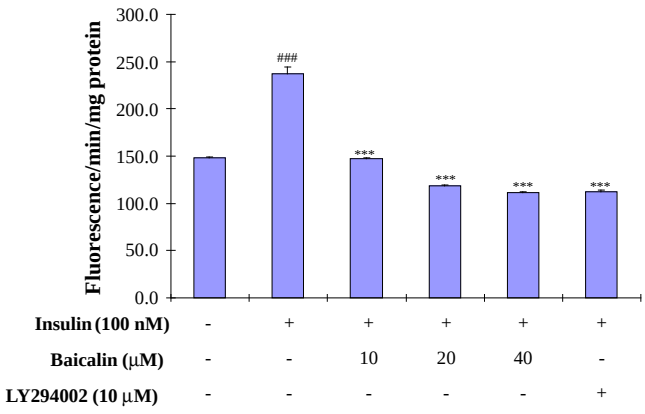

Fig. 7. Baicalin modulated insulin-induced oxidative stress in cultured cell. Quantitative analysis of fluorescence intensity using DCFDA after treatment with vehicle or 100 nM insulin in the absence or in the presence of PI3K/Akt inhibitor (LY294002, 10 μM) and baicalin (10, 20 and 40 μM) for 1 h. As a statistical significance, results of one-factor ANOVA: $^{\#\#}P<.01$ vs. insulin-untreated group; $^{***}P<.001$ vs. insulin-treated group, respectively.

Our finding on SIRT1 is important because SIRT1 is a selective activator of FoxO signaling. The interplay between FoxO and SIRT1 potentiates the resistance to oxidative stress and enhances cell cycle arrest, two properties that promote cellular survival and longevity of the organism [38]. Therefore, SIRT1 can enhance the nuclear translocation and trapping of FoxO1 [39] and probably promote target gene-specific transcription [40]. However, Alcendor et al. [20] reported that moderate overexpression of SIRT1 protects the heart from oxidative stress induced by Paraquat, with increased expression of antioxidants, such as catalase, through FoxO-dependent mechanisms.

Recently, plant polyphenols such as resveratrol have been touted in the promotion of health by the mitigation of many diseases. Resveratrol, which is an activator of SIRT1, remains within the nucleus, overcoming the effect of insulin, probably because insulin-induced phosphorylation of FoxO1 is abolished [39]. Furthermore, resveratrol has been reported to extend life span in yeast through sirtuin activation [41], but did not affect the survival of mice on diet compared to mice on the same diet with

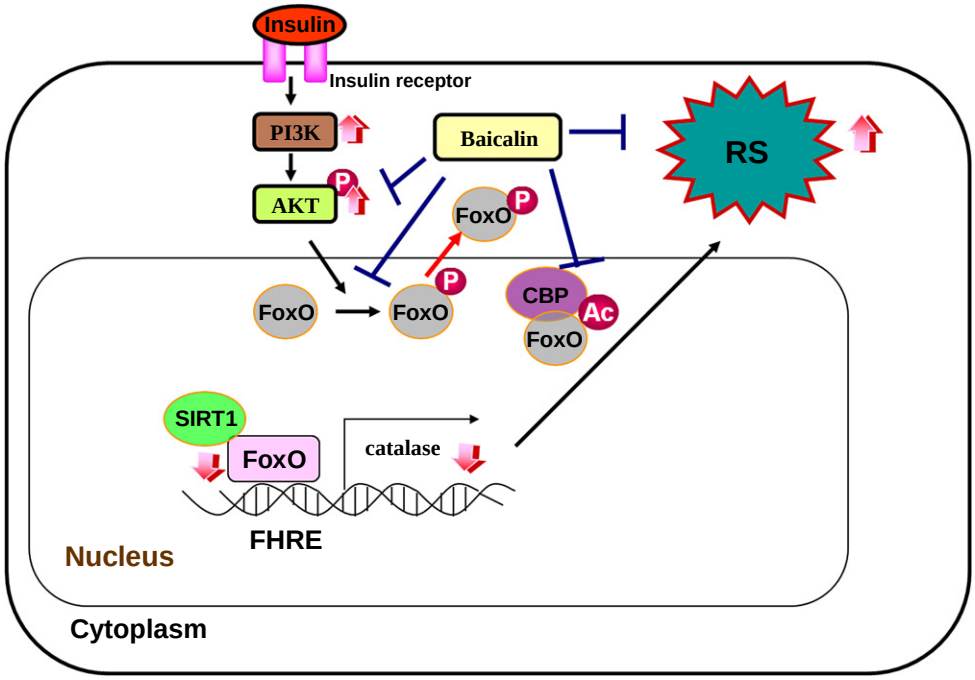

Fig. 8. Possible mechanisms of aging and baicalin in phosphorylation and acetylation of FoxO pathway.

resveratrol [42]. There is a possibility that the efficacy of the baicalin treatment observed in the current study may be related to its potent scavenging action of RS, as suggested by Hung et al. [43] FoxO transcription factors are emerging as master signaling integrators that translate various environmental stimuli into dynamic gene expression programs that influence many physiological and pathological processes, including aging.

To substantiate our data generated from the rat kidney experiments, we examined molecular events related to FoxO1 via the PI3K/Akt pathway in the cultured HEK293T cell system. When cells were exposed to insulin, the PI3K/Akt pathway was activated, resulting in the acetylation and phosphorylation of FoxO1 (Thr 24, Ser 256), and the down-regulation of target gene expression and SIRT1 in deacetylase (Fig. 6A). These data strongly indicate that Akt-mediated phosphorylation and acetylation of FoxO1 were stimulated by insulin. However, baicalin treatment dramatically suppressed insulin-induced phosphorylation and acetylation of FoxO1 (Fig. 6B).

Thus, based on our data and those of others, we have proposed a possible mechanism for baicalin (shown in Fig. 8). Phosphorylated and acetylated FoxO1 down-regulates catalase, thereby weakening the antioxidative defenses of old rats. The gene expression was down-regulated by modulating Akt-mediated phosphorylation and CBP-related acetylation of FoxO1. In this regard, our findings share similar properties of resveratrol, which was shown to up-regulate genes involved in the antioxidant pathway [44].

The significance of the current findings is that the observed age-related decrease in FoxO1 transcriptional activity was due to increased phosphorylation, acetylation and PI3K/Akt during aging, all of which may be related closely to increased levels of insulin with age. More importantly, within the context of aging, theses age-related changes were counteracted by the antiaging action of baicalin.

Based on the data we obtained from the current study, we report for the first time that short-term feeding of baicalin modulated age-related phosphorylation of FoxO1 through PI3K/Akt signaling and FoxO1 acetylation by interacting with CBP and SIRT1 during aging.

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