

## Attenuation of age-related changes in FOXO3a activity and the PI3K/Akt pathway by short-term feeding of ferulate

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**Abstract** Ferulate (4-hydroxy-3-methoxycinnamic acid) is a well-known phenolic compound that scavenges free radicals and exerts anti-inflammatory effects. Forkhead box O3a (FOXO3a), a transcription factor that plays important roles in aging processes, decreases with age and is negatively regulated

through phosphorylation by phosphatidylinositol 3-kinase (PI3K)/Akt signaling. The present study investigated the efficacy of short-term ferulate feeding on age-related changes in PI3K/Akt/FOXO3a and upstream insulin signaling pathways in aged rats. In addition, changes in manganese superoxide dismutase (MnSOD) and catalase expression were examined because of their dependence on PI3K/Akt/FOXO3a activity. Short-term feeding experiments were done with a diet containing ferulate that was given to aged rats at doses of 3 or 6 mg kg<sup>-1</sup> day<sup>-1</sup> for 10 days. Results showed that FOXO3a activity was increased in the ferulate-fed old group compared with the control old group. Also, ferulate suppressed the PI3K/Akt signaling pathway that is responsible for FOXO3a inhibition in aged rats. Plasma insulin levels and the upstream insulin signaling pathway were also modulated by ferulate correspondingly with PI3K/Akt/FOXO3a activity. The age-related decrease in two major antioxidant enzymes, MnSOD and catalase, was blunted by ferulate, which was accompanied by FOXO3a transcriptional activity. The significance of the present study is the finding that short-term feeding of ferulate effectively modulates age-related renal FOXO3a, PI3K/Akt and insulin signaling pathways, and MnSOD and catalase expression, all of which may be beneficial for attenuating the aging process.

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## Introduction

Ferulate (4-hydroxy-3-methoxycinnamic acid) is a phenolic compound ubiquitously found in fruits, grains, and vegetables such as rice bran, tomatoes, and sweet corns (Mathew and Abraham 2004; Ou and Kwok 2004; Fig. 1). An increasing number of reports show the functional efficacies of ferulate including its antioxidant, anti-inflammatory, antithrombotic, anti-diabetic, and anticancer activities (Barone et al. 2009). However, only a limited number of studies report on the effect of ferulate in the modulation of the signaling pathways and transcription factors described below.

Mitogen-activated protein kinases (MAPKs) and the PKB/Akt signaling pathway are two signaling pathways that are reported to be regulated by ferulate under various conditions. Sodium ferulate is shown to prevent amyloid beta-induced mitogen-activated protein kinase kinase (MKK) 3/MKK6-p38 MAPK activity in rat hippocampus (Jin et al. 2006), showing the suppression of the age-related Jun N-terminal kinase (JNK) pathway and upregulation of mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK)1/2 pathway in aged rat hippocampus (Jin et al. 2008). In human umbilical vein endothelial cells (HUVECs), ferulate blocked the JNK pathway, but did not change the ERK (Ma et al. 2010a). The activation of phosphatidylinositol 3-kinase (PI3K)/Akt by ferulate has also been reported in HUVECs and breast cancer MCF7 cells (Chang et al. 2006; Ma et al. 2010b). Previously, we reported that ferulate effectively suppresses redox-sensitive, pro-inflammatory NF-kappaB activation via NF-kappaB-inducing kinase/

IkappaB kinase and MAPKs by reducing oxidative stress in aged rats (Jung et al. 2009).

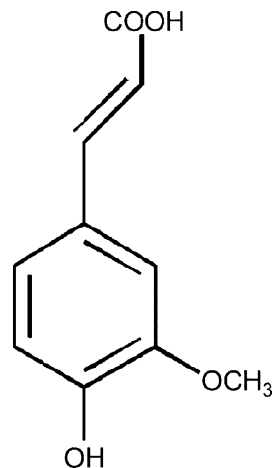
Oxidative stress is considered as a major factor contributing to the aging process and leads to aberrant signaling pathways (Yu and Yang 1996). Aged rats are in the condition of increased oxidative stress and weakened antioxidative defense systems. Therefore, to restrain the aging process, we need to seek ways to attenuate oxidative stress and strengthen the antioxidant system or to modulate the altered signaling pathway molecules.

FOXO3a is one of the transcriptional factors playing such a role in modulating the aging process. It has been suggested that increased FOXO3a activity strengthens the antioxidant defense systems that have led to extended longevity in several experimental organisms (Lin et al. 1997; Ogg et al. 1997). FOXO3a is an isotype of FOXO transcription factors, and it directly induces the antioxidant enzymes manganese superoxide dismutase (MnSOD) and catalase (Storz 2011). FOXO3a binding sites have been identified in promoters of these enzymes (Kops et al. 2002; Li et al. 2008; Tan et al. 2008).

FOXO proteins are largely regulated through the phosphorylation of specific amino acids by Akt, a downstream kinase of PI3K that acts in response to insulin or several growth factors and leads to the translocation of these proteins from the nucleus into the cytoplasm (Salih and Brunet 2008). Three putative Akt-sensitive phosphorylation sites are present in FOXO3a: Thr<sup>32</sup>, Ser<sup>253</sup>, and Ser<sup>315</sup> (Brunet et al. 1999). The phosphorylation of Thr<sup>32</sup> and Ser<sup>253</sup> inhibits the stimulatory effect of FOXO3a upon gene transcription (Brunet et al. 1999).

The PI3K/Akt/FOXO3a pathway is a pivotal signaling pathway regulated by insulin. Aging is known to cause increased insulin and IGF-1 plasma levels (Coschigano et al. 2003). Evidence shows that the suppression of insulin signaling can extend life span in both invertebrate and vertebrate models (Gems et al. 1998; Larsen et al. 1995; Tissenbaum and Ruvkun 1998). Binding of insulin to its cognate receptor leads to the phosphorylation of the receptor itself and adaptor molecule, and insulin receptor substrate (IRS) 1 on tyrosine residues. This in turn activates PI3K, which results in increased levels of phosphatidylinositol 3,4-diphosphate (PIP<sub>2</sub>) and phosphatidylinositol 3,4,5-triphosphate (PIP<sub>3</sub>; Paez and Sellers 2003). This event recruits Akt into the plasma membrane and leads to the phosphorylation of Thr<sup>308</sup> and Ser<sup>473</sup> in Akt by

**Fig. 1** Chemical structure of ferulic acid



PIP<sub>3</sub>-dependent kinase-1 and -2 (PDK-1 and PDK-2), respectively (Alessi et al. 1996; Kitamura et al. 1998). PDK-1 phosphorylates Thr<sup>308</sup> at Akt, and it is activated by the lipid products of PI3K, whereas PDK-2 has not yet been characterized (Alessi et al. 1996; Kitamura et al. 1998; Kops and Burgering 1999). Signaling by PI3K is counterbalanced by the tumor suppressor protein PTEN (phosphatase and tensin homologue deleted on chromosome 10). PTEN dephosphorylates PIP<sub>2</sub> and PIP<sub>3</sub> and prevents the subsequent activation of serine/threonine protein kinases of Akt (Vanhaesebroeck and Alessi 2000; Vanhaesebroeck and Waterfield 1999).

Some natural compounds were found to modulate the PI3K/Akt/FOXO3a pathway, such as epigallocatechin-3-gallate, resveratrol, luteolin, and isoflavone (Belguise et al. 2007; Eddy et al. 2007; Fang et al. 2007; Park et al. 2009; Rice et al. 2007; Zhang et al. 2009). However, to our knowledge, the effect of ferulate specifically on the PI3K/Akt/FOXO3a signaling pathway has not been reported, particularly in the area of aging studies. Therefore, considering the importance of the role of FOXO3a in the aging process, our data provide molecular insights into ferulate as a possible intervention of the aging process.

In the present study, we investigated molecular events related to decreased FOXO3a activity and its regulatory signaling pathway, PI3K/Akt, and their modulation by ferulate in aged rats. Also, plasma insulin levels and the upstream insulin signaling pathway were analyzed, which are known to be responsible for PI3K/Akt/FOXO3a activity. This report further shows that short-term feeding of ferulate has a beneficial effect on the activation of the downstream genes, catalase and MnSOD, in these aged rats.

## Materials and methods

### Animals and ferulate feeding

Young (9 months) and old (21 months) specific pathogen-free male Sprague–Dawley rats were obtained from Samtako (Osan, Korea) and were fed a diet of the following composition: 21% soybean protein, 15% sucrose, 43.65% dextrin, 10% corn oil, 0.15%  $\alpha$ -methionine, 0.2% choline chloride, 5% salt mix, 2% vitamin mix, and 3% Solka-Floc. Old rats were divided into three groups, with  $n=6$ /group. Chow was supple-

mented with ferulate and fed to the 21-month-old group at a dose of either 3 or 6 mg kg<sup>-1</sup> day<sup>-1</sup>. The dose was based on our published earlier study (Jung et al. 2009).

After 10 days, the rats were killed and the kidneys were quickly removed and rinsed in iced-cold buffer [100 mM Tris, 1 mM EDTA, 0.2 mM phenylmethylsulfonylfluoride (PMSF), 1 M pepstatin, 2 M sodium orthovanadate (pH 7.40)]. Tissues were immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

### Materials

All chemical reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA), except where noted. The radionucleotide [ $\gamma$ -<sup>32</sup>P]-ATP (250  $\mu\text{Ci}$ ) and Western blotting detection reagents were obtained from Amersham (Bucks, UK). Antibodies against FKHL1, p-FKHL1 (Thr32), p-FKHL1 (Ser253), catalase, MnSOD, IRS1, p-IRS1 (Tyr632),  $\beta$ -actin, Histone H1, TFIIB, and PI3K (p85 $\alpha$ ) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against p-Akt (Ser473), p-Akt (Thr308), p-PDK1 (Ser241), PDK1, PTEN, and p-PTEN (Ser380) were obtained from Cell Signaling (New England BioLabs, Hertfordshire, UK). Anti-rabbit IgG horseradish peroxidase-conjugated antibody and anti-mouse IgG horseradish peroxidase-conjugated antibody were obtained from Amersham. 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).

### Nuclear extract preparation

The tissue was placed in 2 ml of hypotonic buffer A [10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM MgCl<sub>2</sub>, 1 mM DTT, 0.1 mM EDTA, and 0.1 mM PMSF] and homogenized in an ice bath using the Dounce tissue grinder (Wheaton Manufacturers, NJ, USA). To the homogenates, 125  $\mu\text{l}$  of 10% NP-40 solution was added, and the mixture was then centrifuged at  $14,800\times g$  for 30 s. The pelleted nuclei were washed once with 400  $\mu\text{l}$  of buffer A plus 25  $\mu\text{l}$  of 10% NP-40; centrifuged; resuspended in 50  $\mu\text{l}$  of a solution consisting of 50 mM HEPES (pH 7.8), 50 mM KCl, 0.3 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and 10% glycerol; mixed for 20 min; and centrifuged at  $4^{\circ}\text{C}$ ,  $14,800\times g$  for 5 min. The supernatant containing nuclear

proteins was collected and stored at  $-80^{\circ}\text{C}$  in aliquots until electrophoretic mobility shift assay (EMSA) and Western blotting were done.

#### Western blotting

Homogenized samples were boiled for 5 min with a gel-loading buffer (125 mM Tris-Cl, 4% SDS, 10% 2-mercaptoethanol, pH 6.8, 0.2% Bromophenol Blue) at ratio of 1:1. Thirty micrograms of proteins for each sample was separated by SDS-PAGE using acrylamide gels as described by Laemmli (1970) and transferred to PVDF membrane at 80 V for 2 h in a wet 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  $25^{\circ}\text{C}$  for 1 h, followed by a horseradish peroxidase-conjugated secondary antibody at  $25^{\circ}\text{C}$  for 1 h. Antibody labeling was detected using enhanced chemiluminescence as per the manufacturer's instructions. Pre-stained protein markers were used for molecular weight determinations.

#### Electrophoretic mobility shift assay

The EMSA method was used to characterize the binding activities of FOXO in nuclear extracts (Kerr 1995). FOXO oligonucleotide was 5'-TTA GTC ATT TTG TTT GTT CAT A-3'. Protein-DNA binding assays were performed with 10 g of nuclear protein. To minimize the effect of salt on binding, the concentration of salt was adjusted to the same level in all samples. Nonspecific binding was blocked using 1 g of poly(dI-dC)·poly(dI-dC). The binding medium contains 5% glycerol, 1 mM  $\text{MgCl}_2$ , 50 mM NaCl, 0.5 mM EDTA, 2 mM DTT 1% Nonidet P40 (NP40), and 10 mM Tris/HCl, pH 7.5. In each reaction, 20,000 cpm of a radiolabeled probe was included. Samples were incubated at room temperature for 20 min, and the nuclear protein/ $^{32}\text{P}$ -labeled oligonucleotide complex was separated from free  $^{32}\text{P}$ -labeled oligonucleotide by electrophoresis using 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  $-80^{\circ}\text{C}$ .

#### Insulin assay

Insulin level was determined using a rat insulin ELISA kit (Shibayagi Co., Japan). One hundred microliters of biotin-conjugated anti-insulin solution was added to 10  $\mu\text{l}$  of sample in each well. The chromogenic substrate solution was added to each well after the addition of HRP-conjugated avidin solution to the well. After 30 min of incubation at room temperature, 100  $\mu\text{l}$  of the reaction stopper was added. The optical density was measured at 450 nm using a microplate reader (TECAN, Salzburg, Austria).

#### Statistical analysis

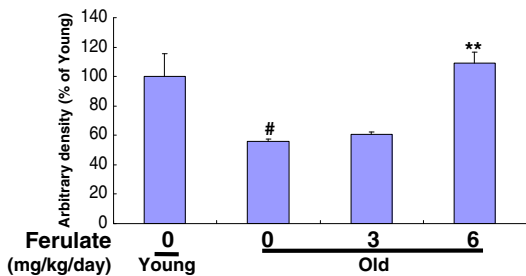
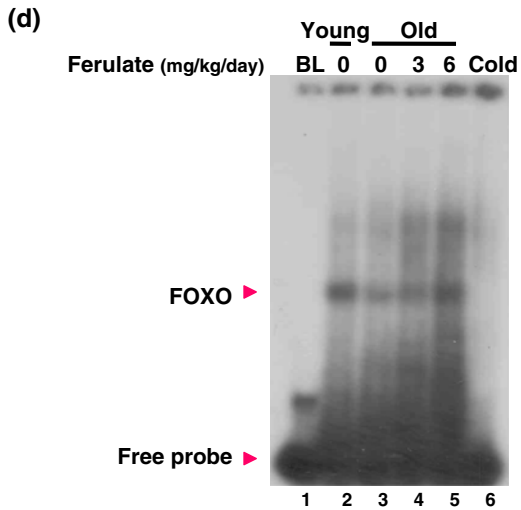
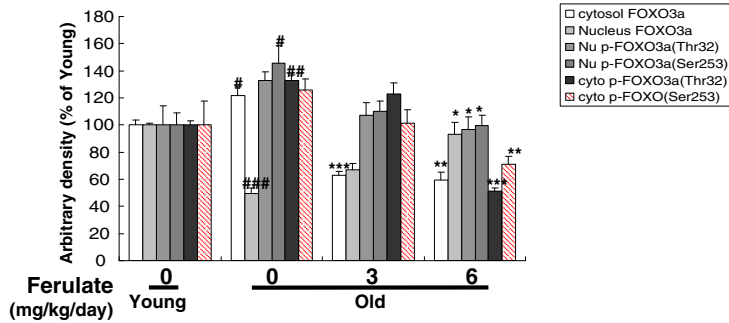
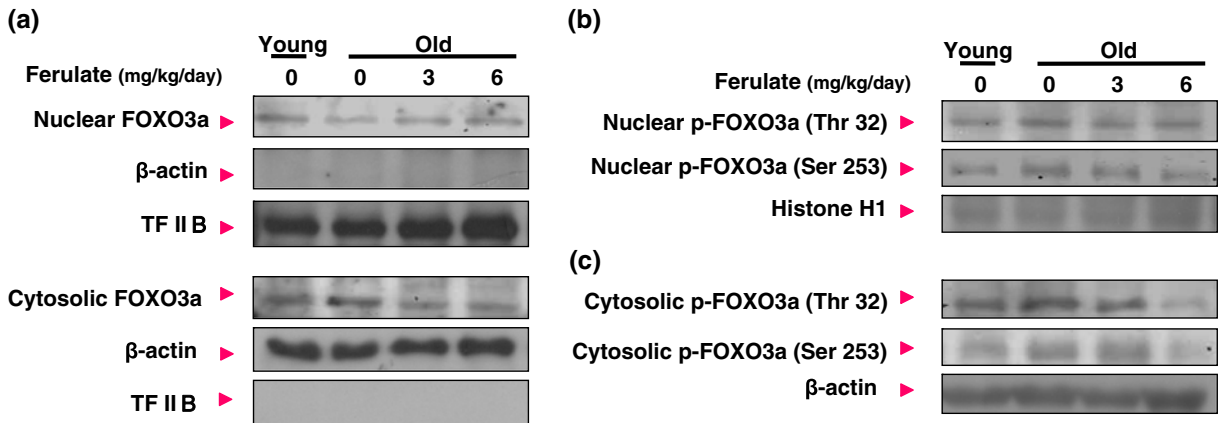
ANOVA was conducted to analyze significant differences among all groups. Differences among the means of individual groups were assessed by Fisher's protected LSD post hoc test. Values of  $p < 0.05$  were considered statistically significant.

## Results

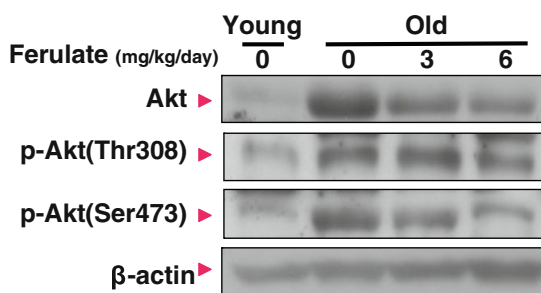
#### Effect of ferulate on FOXO3a phosphorylation and transcriptional activity

The effects of ferulate on FOXO3a activity in aged rats were examined. Aspects of FOXO3a activity were confirmed: translocation to nucleus, phosphorylation, and DNA binding. First, when comparing aged

**Fig. 2** Modulation of FOXO3a transcriptional activity in young, old, and ferulate-fed old rats. Western blot analysis was performed to detect the expressions of cytosolic FOXO3a and nuclear FOXO3a (a). Nuclear  $\beta$ -actin blot and cytosolic TFIIIB blot was shown to clarify the cellular component subfraction (a). The phosphorylation of FOXO3a at Thr<sup>32</sup> and Ser<sup>253</sup> in nuclear fraction (b) and in cytosolic fraction (c) of kidney from young (9 months), old (21 months), and ferulate-fed old rats (a couple of groups of ferulate-fed old rats were fed 3 or 6 mg  $\text{kg}^{-1}$  day<sup>-1</sup> for 10 days, respectively) was measured. The FOXO binding activities were confirmed by the EMSA method in kidney nuclear protein from young, old, and ferulate-fed old rats (d). Lane 1 Probe without nuclear protein sample (BL). Lanes 2–5 Kidney nuclear protein samples of young, old, and ferulate-fed old rats. Lane 6 Competition assay using a 100-fold excess of unlabeled FHRE oligonucleotide. The blots were quantified by densitometry as a percentage of the level of 9-month-old rats. As statistical significance, results of one-factor ANOVA were used: <sup>#</sup> $p < 0.05$ , <sup>##</sup> $p < 0.01$ , <sup>###</sup> $p < 0.001$  vs. young rats; \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. old non-ferulate-fed rats, respectively



rats with their young counterparts, nuclear total FOXO3a decreased in contrast to the increase in cytoplasmic total FOXO3a, which implies the translocation of FOXO3a to the cytosol from the nucleus. However, ferulate effectively countered this translocation of FOXO3a from the nucleus in aged rats (Fig. 2a). Also, ferulate changed the phosphorylation levels of FOXO3a in aged rats. As mentioned above, phosphorylated FOXO3a at Thr<sup>32</sup> and Ser<sup>253</sup> could no longer be located in the nucleus, which would lead to the inhibition of the transcriptional activity (Brunet et al. 1999). We found that the phosphorylation of these sites of nuclear FOXO3a was increased in the untreated old rats compared with the young, but the ferulate-fed old groups showed a dose-dependent, lower age-related phosphorylation of nuclear FOXO3a at Thr<sup>32</sup> and Ser<sup>253</sup> (Fig. 2b). These data correspond with the data on the changes in total FOXO3a localization. The phosphorylation of FOXO3a at these sites in the cytoplasm also increased during aging and was blocked by the dietary ferulate treatment (Fig. 2c). Finally, to determine the effect of ferulate on the age-related DNA binding activity of FOXO, EMSA was carried out using nuclear proteins. The nuclear binding activity of FOXO was shown to decrease with age. However, the ferulate-fed old rats showed increased DNA binding activity of FOXO compared with untreated old rats (Fig. 2d). The binding specificity of the FOXO complex was demonstrated using a 100-fold excess of an unlabeled oligonucleotide, which competed for binding (Fig. 2d, lane 6).



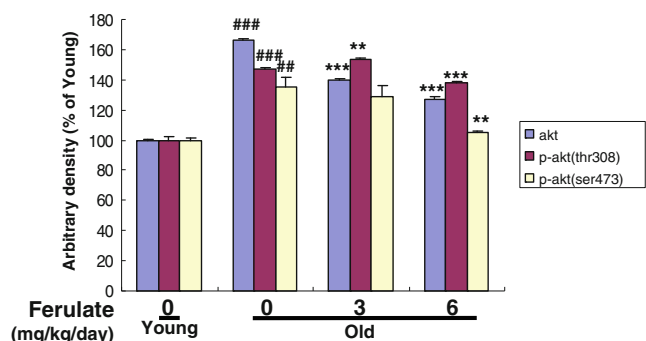
**Fig. 3** Effect of ferulate on Akt protein levels and phosphorylated Akt. Western blot analysis was performed to detect the total protein level and phosphorylated forms of Akt at Thr<sup>308</sup> and Ser<sup>473</sup>, reflecting the activity of Akt, in kidney homogenate from young (9 months), old (21 months), and ferulate-fed old rats (a couple of groups of ferulate-fed old rats were fed 3 or

### Effect of ferulate on Akt activity

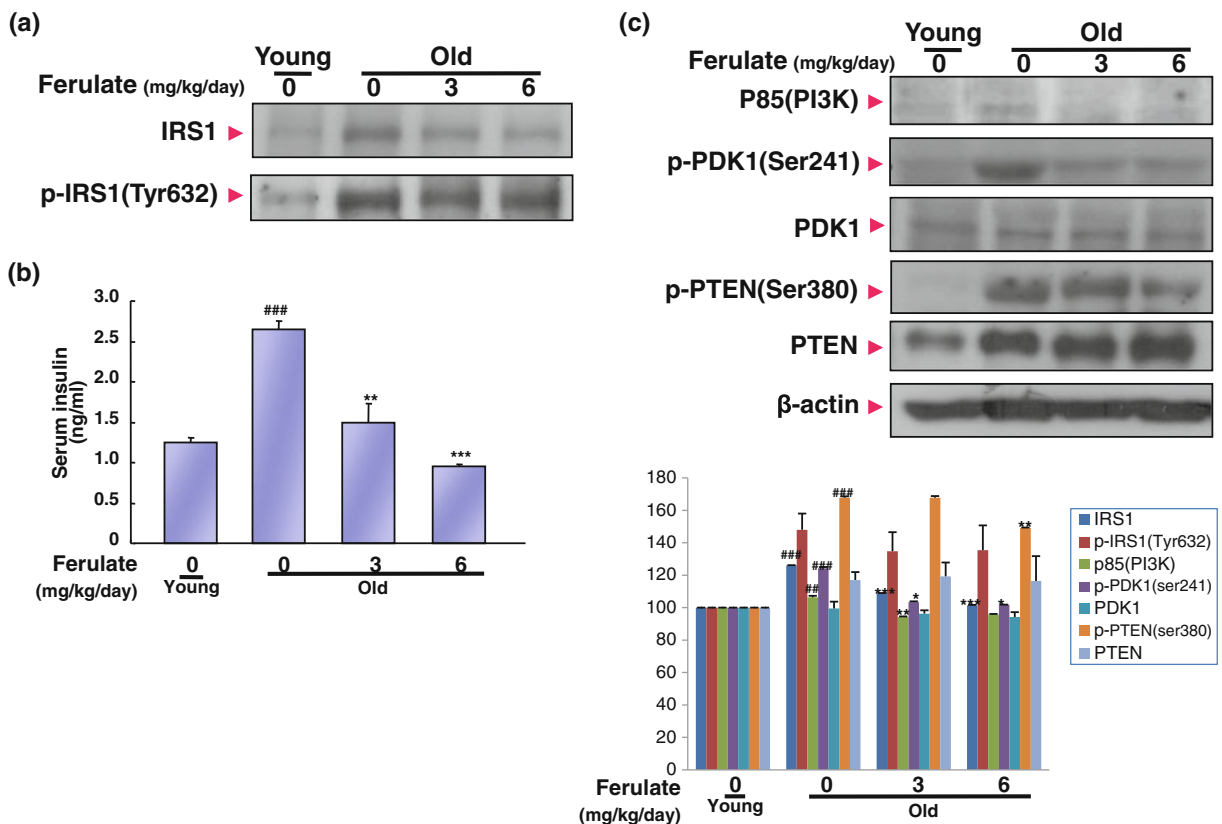
We analyzed the expression and activity of renal Akt which plays a major role in the regulation of FOXO3a. The activity was measured as a phosphorylated form at Thr<sup>308</sup> or Ser<sup>473</sup>. The total protein level of renal Akt increased with age, but dose-dependently decreased with ferulate treatment. Phosphorylated Akt showed the same result in total protein levels (Fig. 3). These data correspond with the results found in FOXO3a phosphorylation.

### Effect of ferulate on insulin signaling and plasma insulin level

We wanted to examine the effect of ferulate on the status of insulin receptor substrate-1 (IRS-1), the key transmitting upstream molecule that modulates the PI3K/AKT pathway. IRS is phosphorylated by an activated insulin receptor and then provides a docking site to p85, a subunit of PI3K. Ferulate treatment was shown to inhibit the protein level and phosphorylation of renal IRS-1 with age (Fig. 4a). As insulin is mainly responsible for the activation of IRS-1, we analyzed the effect of ferulate on plasma insulin levels. The plasma insulin level increased in aged rats compared with the young group, as we previously showed (Fig. 4b; Kim et al. 2008). Ferulate effectively reduced the age-related increase of plasma insulin level, which corresponded with IRS activation (Fig. 4b).



6 mg kg<sup>-1</sup> day<sup>-1</sup> for 10 days, respectively). The blots were quantified by densitometry as a percentage of the level of 9-month-old rats. As a statistical significance, results of one-factor ANOVA were used: ##*p*<0.01, ###*p*<0.001 vs. young rats; \*\**p*<0.01, \*\*\**p*<0.001 vs. old non-ferulate-fed rats, respectively



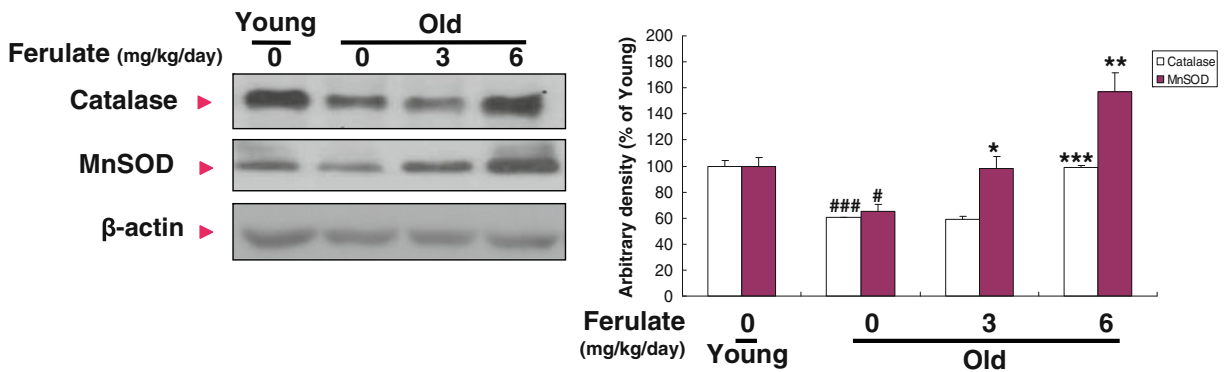
**Fig. 4** Modulation of age-related increases in insulin signaling pathway activity and plasma insulin level by ferulate. Western blot analysis was performed to detect IRS1 and phosphorylated IRS1 at Tyr<sup>632</sup> (a). The plasma insulin level was analyzed by a chemical reagent kit (b). Subsequently, downstream signaling molecules including p85 (a subunit of PI3kinase), phosphorylated PDK1 at Ser<sup>241</sup>, PDK, PTEN, and phosphorylated PTEN at Ser<sup>380</sup> (c) were

measured in kidney homogenate from young (9 months), old (21 months), and ferulate-fed old rats (a couple of groups of ferulate-fed old rats were fed 3 or 6 mg kg<sup>-1</sup> day<sup>-1</sup> for 10 days, respectively). The blots were quantified by densitometry as a percentage of the level of the 9-month-old rats. As statistical significance, results of one-factor ANOVA were used: ##  $p < 0.01$ , ###  $p < 0.001$  vs. young rats; \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  vs. old non-ferulate-fed rats, respectively

Subsequently, downstream molecules of IRS were investigated. Ferulate inhibited the age-induced increase of p85 in kidney (Fig. 4c). Phosphorylated PDK1 in its activated form directly phosphorylates Akt. Ferulate suppressed the p-PDK1 (Ser<sup>241</sup>) increase with age, but did not affect the total PDK1 (Fig. 4c). Inactivation of a negative regulator of insulin signaling PTEN was shown to increase with age, but ferulate suppressed p-PTEN (Ser<sup>380</sup>; Fig. 4c). Total PTEN level was increased in aged rats, but ferulate did not have a significant effect on the total PTEN (Fig. 4c). Taken together, ferulate was able to effectively modulate the upstream regulators and the inducer insulin of PI3K/Akt activation in aged kidney.

Effect of ferulate on the expressions of catalase and MnSOD

To confirm the activation of FOXO3a by ferulate supplementation, the expressions of MnSOD and catalase were examined by Western blot analysis (Fig. 5). As mentioned above, MnSOD and catalase are obvious downstream target genes of FOXO3a, and their expression levels are well known to decrease during the aging process (Rao et al. 1990; Tian et al. 1998). In this present study, the protein levels of these enzymes were decreased in the old animals compared with the young counterparts. However, old rats receiving dietary ferulate showed significant and concentration-dependent increases in the protein



**Fig. 5** Expression levels of renal MnSOD and catalase, downstream targets of FOXO3a. Western blot analysis was performed to detect the expression levels of MnSOD and catalase, measured in kidney homogenates from young (9 months), old (21 months), and ferulate-fed old rats (a couple of groups of ferulate-fed old rats were fed 3 or 6 mg kg<sup>-1</sup> day<sup>-1</sup>

for 10 days, respectively). The blots were quantified by densitometry as a percentage of the level of 9-month-old rats. As a statistical significance, results of one-factor ANOVA were used: #*p*<0.05, ###*p*<0.001 vs. young rats; \**p*<0.05, \*\**p*<0.01, \*\*\**p*<0.001 vs. old non-ferulate-fed rats, respectively

levels of MnSOD and catalase compared to their non-treated old counterparts. The old rats fed a high dose of ferulate showed MnSOD and catalase levels similar to young rats.

## Discussion

In the present study, we obtained evidence showing the ability of ferulate to modulate the PI3K/Akt/FOXO3a pathway, which was accompanied by the induction of MnSOD and catalase in aged rats. Also, it was shown that the age-related increase in plasma insulin level and the activation of renal insulin signaling pathway were counteracted by ferulate treatment.

Ferulate is known to have anti-diabetic, antithrombotic, anti-inflammatory, anticancer, and anti-aging effects (Barone et al. 2009; Mandal et al. 2008). In most studies, ferulate has been explored largely as an antioxidant based on its phenolic structure, through which it scavenges free radicals by its hydroxyl and phenoxy groups that readily donate electrons (Graf 1992; Kanski et al. 2002). Recently, many researchers who are interested in ferulate have looked into its functionality as a modulator of signal transduction and gene expression. Here in the present study, we uncovered some of the molecular modulations underlying the role of dietary ferulate in altering signaling pathways and transcription factors in aged rats.

One of our interesting findings is that a 10-day ferulate feeding to aged rats suppressed the age-related reduction of renal FOXO3a activity by retaining it in the nucleus. Li et al. (2006) reported that the phosphorylation and inactivation of FOXO3a increased in the vascular smooth muscle cell of old rats. In our experiments with aged kidney, increased phosphorylation of FOXO3a and its inactivation was downregulated by ferulate by preventing its transport from the nucleus to the cytoplasm. Our data further provide information on the regulatory signaling pathway responsible for the phosphorylation of FOXO3a. The PI3K/Akt and upstream signaling pathways induced in aged kidney resulted in FOXO3a inhibition, but ferulate effectively suppressed these inductions. At present, little is known about the effect of ferulate on the modulation of the age-related insulin and PI3K/Akt signaling pathways. Although ferulate was reported to modulate hippocampal MAPK and PI3K/Akt in aged rats (Jin et al. 2008), at present, no available data show the age-related change in the insulin pathway by ferulate.

Our data showed changes in total Akt levels as well as phosphorylated forms. In aged rats, Akt activity, as shown by phosphorylated forms, was upregulated, and its upstream signaling pathway corresponded with this upregulation. In a previous study, we showed that an enhanced Akt/PI3K signaling pathway in aged rats by calorie restriction has a potent anti-aging effect (Kim et al. 2008). Several studies have reported on various Akt expression



levels in different aged tissues. For example, both Akt protein levels and activity are generally reduced in insulin-resistant or aged muscle (Wu et al. 2011). Another reported that phosphorylation of Akt at Ser<sup>473</sup> and Thr<sup>308</sup> was higher in the soleus muscles of very old rats (33 months) compared with 6- and 27-month-old rats (Wu et al. 2009). In the hippocampus, the total Akt level increased and phosphorylated Akt expression was unaltered in 24-month-old rats compared to 3-month-old rats (Janner et al. 2010). Unlike the hippocampus, the hypothalamus of 24-month-old rats showed an enhanced total Akt level after increased phosphorylation, while no significant alterations in total and phosphorylated Akt were found in 18-month-old animals (Janner et al. 2010).

The increase of two major antioxidant enzymes, MnSOD and catalase, in the ferulate-fed rats is interesting because of the protective role these antioxidants play in maintaining redox balance against oxidative stress and other deleterious actions of aging. It has been shown that FOXO3a binds to the promoter region of MnSOD and catalase and transcriptionally controls the expression of these enzymes (Kops et al. 2002; Tan et al. 2008). Our data on FOXO3a indicate that the increased FOXO3a activity by ferulate is likely responsible for the enhancement of these antioxidative enzyme defenses against increased oxidative stress during aging. Other researchers have reported that ferulate feeding or cell treatment with ferulate increases the activity of MnSOD and catalase, however without clear molecular delineation.

One other important point worth commenting on is the modulation of PI3K/Akt/FOXO3a by ferulate. The PI3K/Akt signaling pathway is activated by both oxidative stress and insulin. As mentioned earlier, ferulate suppressed increased oxidative stress in aged rats, as reported from our laboratory (Jung et al. 2009). As shown in Fig. 4 of the present study, ferulate modulated the insulin level in aged rats. This reduced insulin level might affect the PI3K/Akt/FOXO3a pathway to decrease because insulin is a well-known stimulant for FOXO3a activity. Thus, it is possible that the decreased PI3K/Akt/FOXO3a we observed in the current study could be a net result of the combined effect of the antioxidative action of ferulate and its insulin-lowering action, signifying that ferulate is an effective bioactive substance with diverse effects. Further study is needed to clarify the

exact molecular mechanisms underlying the interaction of ferulate and PI3K/Akt/FOXO3a in aged rats. Ferulate has been reported to increase plasma insulin levels in a model of *db/db* mice (Jung et al. 2007). Different results might come from different circumstances, or pathogenic and normal models.

Putting the data together, dietary ferulate was shown to increase FOXO3a activity and modulate its regulatory insulin signaling pathway in kidney from aged rats. This is the first report to examine the effects of short-term dietary ferulate on the age-related change in the PI3K/Akt/FOXO3a signaling pathway *in vivo*. We provide evidence for ferulate as a novel inducer of FOXO3a activation and antioxidative defense enzymes and as a modulator of its own regulatory signaling pathway, all of which influence the aging process.

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