The essential role of FoxO6 phosphorylation in aging and calorie restriction

Dae Hyun Kim · Min Hi Park · Ki Wung Chung · Min Jo Kim · Yu Ri Jung ·
Ha Ram Bae · Eun Ji Jang · Jun Sik Lee · Dong Soon Im · Byung Pal Yu ·
Hae Young Chung

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Abstract Changes in the activities of FoxOs caused by phosphorylation, acetylation, or ubiquitination induce expressional changes in the genes involved in the modulation of oxidative stress by modifying histones and chromatin and can substantially alter cellular functions during aging and age-related diseases. However, the precise role that FoxO6, a novel member of the FoxO class of transcription factors, plays in the aging kidney has not been determined. The purpose of this study was to determine the role played by FoxO6 in the

maintenance of redox homeostasis in HEK293T cells and aged kidney tissues isolated from ad libitum (AL)-fed and 40 % calorie restriction (CR) rats. The results obtained from AL-fed rats showed that diminished FoxO6 activity during aging was caused by FoxO6 phosphorylation, which disabled its transcriptional activity. In contrast, CR rats were found to have significantly higher FoxO6 activities and maintained redox balance. To determine the molecular mechanism responsible for FoxO6 modification by age-related oxidative stress, we examined H₂O₂-treated HEK293T cells in which FoxO6 was inactivated by phosphorylation and found that H₂O₂-induced oxidative stress promoted FoxO6 phosphorylation via PI3K/Akt signaling. The results of this study show that the protective role of FoxO6 in the aging process may in part be related to its ability to attenuate oxidative stress by upregulating catalase expression, as shown in CR. This delineation of the role of FoxO6 expands understanding of the pathological and physiological mechanisms of aging.

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D. H. Kim · M. H. Park · K. W. Chung · M. J. Kim ·
Y. R. Jung · H. R. Bae · E. J. Jang · D. S. Im · H. Y. Chung
Molecular Inflammation Research Center for Aging
Intervention (MRCA), College of Pharmacy, Pusan National
University, Gumjung-gu, Busan 609-735, Republic of Korea

J. S. Lee
Department of Biology, College of Natural Science,
Chosun University, Gwangju 501-759, Republic of Korea

B. P. Yu
Department of Physiology, The University of Texas Health
Science Center at San Antonio, San Antonio, TX 78229, USA

H. Y. Chung (✉)
Molecular Inflammation Research Center for Aging
Intervention (MRCA), College of Pharmacy, Pusan National
University, San 30, Jangjun-dong, Gumjung-gu,
Busan 609-735, Republic of Korea
e-mail: hyjung@pusan.ac.kr

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Introduction

Many recent investigations have demonstrated that FoxO transcription factors play key roles in the inductions of various downstream target genes, which include regulators of metabolism, cell cycle, cell death, and the

oxidative stress response (Accili and Arden 2004). Furthermore, it has been established that in mammals, the FoxO (Forkhead transcription factor) family consists of the evolutionally highly conserved forkhead transcription factors, FoxO1, FoxO3a, FoxO4, and FoxO6 (Van der Heide et al. 2004).

FoxO family members also play important roles in the aging process (Chung et al. 2011), particular, by suppressing the generation of reactive oxygen species (ROS), although it should be noted that FoxO4 plays a detrimental role by upregulating the expressions of MAFbx and MURF1 during muscle aging (Clavel et al. 2006). The crucial roles played by the FoxO family in downstream of PI3K/Akt pathways that might affect insulin levels and be involved in oxidative stress-related diseases and the aging process are also well appreciated (Karger et al. 2009).

The FoxO is a key epigenetic mechanism in aging (Ni et al. 2012; Ribarič 2012). In response to insulin or oxidative stress, FoxO proteins are phosphorylated by protein kinase B (PKB, also known as Akt), a downstream kinase of phosphatidylinositol 3-kinase (PI3K), and this phosphorylation leads to the translocation of these proteins from the nucleus into the cytoplasm (Brunet et al. 1999). However, another epigenetic mechanism involves the posttranslational acetylations of FoxO3 and FoxO4 (Brunet et al. 1999) by cAMP-response element-binding protein (CREB)-binding protein (CBP). Furthermore, CBP triggers the transactivation functions of FoxO1 and FoxO4, whereas their acetylation by CBP attenuates their transcriptional activities (Brunet et al. 1999). In addition, FoxO4 has been shown to reduce cellular oxidative stress by directly increasing manganese superoxide dismutase (MnSOD) and catalase levels (Fukuoka et al. 2003).

Oxidative stress damages all cellular components, including protein, lipid, and DNA, and thereby, promotes cellular senescence (de Magalhães and Church 2006), compromises cell function, and threatens cell survival (Zanichelli et al. 2012). Thus, oxidative stress can disrupt the normal mechanisms of cellular signaling. Interestingly, intricate interactions between Akt and FoxO have been recently reported in the context of the mechanisms of cellular regulation. For example, in yeast, a mutation of Sch 9, which is homologous to Akt, extended lifespan (Fabrizio et al. 2001), and an insulin receptor mutation that decreased the activity of the insulin/IGF-1-like pathway has been reported to increase longevity in fruit flies (Tatar et al. 2001), mice (Bluhner et al. 2003), and human (de Magalhães et al. 2012).

It is also interesting that these lifespan-extending mutations are associated with increased resistance to oxidative stress, which is partly mediated by the increased expression of antioxidant genes (Honda and Honda 1999). Other investigators have shown that PKB-regulated FoxO can reduce levels of cellular oxidative stress by directly increasing the messenger RNA (mRNA) and protein levels of MnSOD and catalase (Burgering and Medema 2003). However, little information is available on the status of FoxO6 during aging or of its modulation by various signaling factors and calorie restriction (CR), the gold standard of aging intervention.

Previous studies have shown that FoxO6 plays an important role in the regulation of hepatic glucose homeostasis in mice (Kim et al. 2011) and in the development of gastric carcinoma. Furthermore, Chung et al. (2013) reported that FoxO6 and PGC-1 α form a regulatory loop that sets the level of oxidative metabolism in skeletal muscle. However, the role played by FoxO6 in aging has not been well defined.

CR has been shown to delay age-related biologic changes and to suppress a number of age-associated pathologic abnormalities, regardless of sex, in mammalian and nonmammalian species (Yu 2005). Furthermore, CR is known to suppress oxidative-related alterations and oxidative-induced age-related diseases and to extend lifespan (Stepanyan et al. 2006).

In the present study, to obtain a better understanding of the mechanism underlying the oxidative-dependent aging process, we investigated the effect of FoxO6 on oxidative stress in aged kidney. In addition, we explored FoxO6 phosphorylation and the process whereby PI3K/Akt signaling modulates FoxO6 activities during aging by studying HEK293T cells and aged kidney tissue isolated from ad libitum-fed (AL) and 40 % CR rats.

Materials and methods

Animals

Specific pathogen-free male Fischer 344 rats (6 or 24 months old) were obtained from Samtako (Osan, Republic of Korea) and fed a diet of the following composition: 21 % soybean protein, 15 % sucrose, 43.65 % dextrin, 10 % corn oil, 0.15 % α -methionine, 0.2 % choline chloride, 5 % salt mix, 2 % vitamin mix,

and 3 % Solka-Floc fiber. The ad libitum (AL)-fed group had free access to both food and water, but animals in the CR were fed 60 % of the food intake of their AL-fed littermates, beginning at 6 weeks of age.

At prescribed times, rats were fed-conditioned and sacrificed by decapitation, and kidneys were quickly removed, rinsed in iced-cold buffer [100 mM Tris, 1 mM EDTA, 0.2 mM phenylmethyl-sulfonylfluoride (PMSF), 1 μ M pepstatin, 2 μ M sodium orthovanadate (pH 7.4)], immediately frozen in liquid nitrogen, and stored at -80°C . The kidney is metabolically active and sensitive to many age-related changes including redox responsive molecular events, which make the kidney suitable for the study. In addition, our laboratory has substantial experience and data on age-related renal changes. The animal protocol used in this study was reviewed and approved by the Pusan National University-Institutional Animal Care and use Committee (PNU-IACUC).

Cell culture system

Human embryo kidney 293 T cells (HEK293T) cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Nissui Co., Tokyo) supplemented with 10 % heat-inactivated (56 $^{\circ}\text{C}$ for 30 min) fetal bovine serum (Gibco, Grand Island, NY), 233.6 mg/ml glutamine, 100 mg/ml penicillin streptomycin, 0.25 $\mu\text{g}/\text{ml}$ amphotericin B, and 10 % heat-inactivated fetal bovine serum. Cells were maintained at 37 $^{\circ}\text{C}$ in a 5 % CO_2 humidified atmosphere.

Materials

All chemical reagents were obtained from Sigma (St. Louis, MO, USA), except where noted. 2',7'-Dichlorodihydrofluorescein was from Molecular Probes, Inc. (Eugene, OR, USA). Western blotting detection reagents were obtained from Amersham (Bucks, UK). RNazolTM B was obtained from TEL-TEST, Inc. (Friendwood, TX, USA). Antibodies against FoxO1, p-FoxO1 (Thr24), catalase, MnSOD, β -actin, histone H1, p-Akt, and total-Akt were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against p-FoxO1 (Ser256) were obtained from Cell Signaling (New England BioLabs, Hertfordshire, UK). Antibodies against FoxO6, p-FoxO6 (Ser184)

and FoxO6-wt, FoxO6-siRNA, and CA-Akt virus were obtained from Dr. H.H. Dong (University of Pittsburgh, PA). Anti-rabbit IgG-horseradish peroxidase-conjugated antibody and anti-mouse IgG-horseradish peroxidase-conjugated antibody were from Amersham (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).

Nuclear extract preparation

Frozen rat kidney tissues (0.2–0.4 μg) were rinsed in PBS buffer and then transferred to a Dounce tissue grinder (Wheaton Manufacturers, NJ). Solution A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM PMSF) was added at 2.5 ml/g tissue. Five strokes of pestle were used to homogenize tissue to a 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 predominantly cytoplasmic constituents. To obtain a nuclear pellet, 400 μl of solution C (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM of each of EDTA, EGTA, DTT, and PMSF) was added. 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 a microcentrifuge. Supernatants, which contained nuclear proteins, were then removed, transferred carefully to a fresh tube, and stored at -80°C until required for Western blotting. Protein contents were determined using the bicinchoninic acid protein assay (Sigma, St. Louis, MO, USA).

Western blotting

Western blotting was carried out as described previously (Kim et al. 2008). Homogenized samples were boiled for 5 min with a gel-loading buffer (125 mM Tris-HCl, 4 % sodium dodecyl sulfate (SDS), 10 % 2-mercaptoethanol, pH 6.8, 0.2 % bromophenol blue) at a 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 (1970) and transferred to a 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.

Transfection and luciferase reporter assay

Catalase activities were estimated using catalase-Luc vector (Dr. Dong, University of Pittsburgh, PA, USA) that contained a specific binding sequence for FoxO. Transfection was carried out using Lipofectamine 2000 (Invitrogen). Briefly, 1×10^4 cells per well were seeded in 48-well plates. When cultured cells reached about 40 % confluence, they were treated with 1 µg DNA/0.5 µl Lipofectamine 2000 complexes in 500 µl normal media (10 % serum contained) for 24 h and then treated with the virus of FoxO6 (100 multiplicity of infection (MOI)) and FoxO6-siRNA (100 MOI) 24 h after transfection. Subsequently, 100 µM of H₂O₂ was treated for 2 h, and the cells were washed with PBS and subjected to the Steady-Glo Luciferase Assay System (Promega, Madison, WI, USA). Luciferase activity was measured by a luminometer (GENious, TECAN, Salzburg, Austria).

Assay for insulin

Insulin levels were measured using a rat insulin ELISA kit (Shibayagi Co, Japan). Briefly, 100 µl of biotin-conjugated anti-insulin solution was added to 10 µl of sample per well. HRP-conjugated avidin solution and chromogenic substrate solution were then added. After incubation for 30 min at room temperature, 100 µl of reaction stopper was added. Optical densities were measured at 450 nm using a microplate reader (TECAN, Salzburg, Austria).

Reactive species (RS) scavenging activity

RS generation was measured as previously described (Kim et al. 2008) in tissue. One hundred and twenty-five

micromolars of 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) was added to the homogenate with buffer for a final volume of 250 µl.

For the determination of intracellular RS generation activity, 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 FoxO6-wt, FoxO6-siRNA, or CA-Akt virus and were pre-incubated for 1 day. After treatment with H₂O₂ (100 µM) for 2 h, the medium was replaced with a fresh, serum-free medium, and DCFDA (2.5 µM) was added. The fluorescence intensity of DCF was measured every 5 min for 1 h using the microplate fluorescence reader TECAN (Salzburg, Austria) with excitation and emission wavelengths of 485 and 535 nm, respectively.

Immunoprecipitation (IP) of nuclear extracts

Nuclear extracts were immunoprecipitated 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 % NP-40 containing leupeptin (2 µg/ml), aprotinin (1 µg/ml), and pepstatin A (1 µg/ml) (Kim et al. 2008). Aliquots of nuclear extracts were then precleared using a 50 % protein A agarose for 30 min at 4 °C. The nuclear extracts were centrifuged at 12,000×g at 4 °C for 15 min, incubated overnight at 4 °C with the required antibody, and then incubated overnight at 4 °C with 50 % protein A agarose slurry. After washing the immunoprecipitates three times with the IP buffer, the immunoprecipitated proteins were analyzed by SDS-PAGE, and Western blotting analysis was performed as described above.

Immunostaining

HEK293T cells were seeded at 1×10^4 cells per well in a 12-well plate, incubated for 24 h, fixed in 4 % paraformaldehyde solution (15 min at room temperature), washed with PBS buffer, blocked with 3 % normal goat serum (Gibco, Grand Island, USA), and immunostained using rabbit anti-FoxO6 antibody (1:1,000 dilution, Santa Cruz, CA) at 4 °C overnight. Cells were then washed with TBS and incubated for 3 h in the presence of anti-rabbit IgG labeled with Alexa Fluor 488 (1:200; Invitrogen, CA, USA). Cell nuclei were visualized by immunostaining with Hoechst 33342 (1:1,000; Invitrogen), and FoxO6 was determined by confocal laser scanning microscopy (TCS SP2, Leica, Wetzlar, Germany).

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was used to study the interaction between FoxO6 and catalase promoter in HEK293T cells. Cells (1×10^5) were cultured in a medium supplemented with H_2O_2 with or without 100 MOI FoxO6 viruses. After incubation for 24 h, cells were cross-linked with 1 % formaldehyde and ultrasonicated (VCX-600 ultrasonicator; Sonics & Materials Inc., Danbury, CT) at 30 % of maximum power five times for 20 s. After centrifugation at $18,000 \times g$ for 10 min, the supernatant was incubated with 5- μ g polyclonal rabbit anti-FoxO6 antibody that was generated (Kim et al. 2011) and immunoprecipitated using a ChIP assay kit (Upstate Biotechnology, Lake Placid, NY). Immunoprecipitates were analyzed by PCR using catalase promoter-specific primers (GenBank accession number AY545477, 372 bp; forward 5'-GAGCTGAGAAAGCATAGCTATG-3', reverse 5'-CAGCCAATCA GCACCACCCC-3').

Statistical analysis

ANOVA was conducted to analyze significant differences among all groups. Fisher's protected LSD post hoc test was used to determine the significance between group means. Statistical significance was accepted at p value of <0.05 .

Results

FoxO6 activity was downregulated by oxidative stress and aging

It has been reported that in mice, FoxO6 mRNA can be detected in the kidney, lung, and muscle in the fed condition, and that low levels are detectable in the liver (Kim et al. 2011). In the present study, we investigated FoxO6 function in aged kidney tissues.

We found FoxO6 levels were lower in the kidney tissues of old (24 months) rats than in those of young (6 months) rats and that CR animals had higher FoxO6 levels than AL animals of the same age (Fig. 1).

To identify the molecular events underlying H_2O_2 -induced FoxO6 activation in HEK293T cells, Western blot analysis was used to examine the cytosolic translocation of FoxO6. When HEK293T cells were treated with 100 μ M H_2O_2 in serum-free media for 0.5 to 8 h

(Fig. 2a), nuclear FoxO6 protein levels were found to noticeably decrease and cytoplasmic FoxO6 levels were markedly increased 1 h after treatment. Furthermore, when HEK293T cells were treated with H_2O_2 at concentration from 50 to 1,000 μ M for 1 h, FoxO6 protein showed a remarkable shift from the nucleus to the cytoplasm at concentrations above 50 μ M (Fig. 2b). Moreover, RS levels were found to be upregulated in an H_2O_2 concentration-dependent manner (Fig. 2c). These results suggest that oxidative stress regulates FoxO6. In addition, immunostaining showed that treatment with 100 μ M H_2O_2 induced the translocation of FoxO6 from the nucleus to the cytoplasm.

Effects of age and CR on FoxO6 phosphorylation

Because FoxO transcription factors play a central role in the regulation of stress response (Kim et al. 2005), we investigated their modifications during aging. As shown in Fig. 3a, FoxO6 phosphorylation increased as FoxO6 activity decreased during aging, but this imbalance was effectively counterbalanced by CR-induced dephosphorylation.

Furthermore, FoxO6 phosphorylation levels were higher in old rats than in young rats, whereas CR animals had lower FoxO6 levels than AL animals of the same age (Fig. 3b). FoxO1 (Thr24) phosphorylation levels were also higher in old rats, and CR animals showed lower FoxO1 levels than the same aged AL animals (Fig. 3b). These results suggest similar roles for FoxO1 and FoxO6 during aging.

To identify the mechanism responsible for the inability of FoxO6 to undergo subcellular redistribution, we examined the association between FoxO6 and 14-3-3 β , a scaffold protein known to bind FoxO and modify its transcriptional activity (Nielsen et al. 2008). We found increased association between FoxO6 and 14-3-3 β during aging in the control animals and a decreased interaction between FoxO6 and 14-3-3 β in the CR animals (Fig. 3a). These data indicate the incapability of FoxO6 to undergo aging-dependent interaction with 14-3-3 β .

Changes in expression of FoxO6 target genes during aging and CR

Other investigators have shown that Akt-regulated FoxO can reduce the level of cellular oxidative stress

Fig. 1 Effects of FoxO6 on oxidative stress-induced aging and CR. Levels of FoxO6 in young and old, AL and CR rat kidney tissues as assessed by Western blotting ($n=6$ in each group). One representative result for each protein is shown of three experiments that yielded similar results. Results of one-factor ANOVA: $*p<0.05$ vs. young AL or CR rats. *Y* young (6-month-old), *O* old (24-month-old), *AL* ad libitum, *CR* calorie restriction

by directly increasing protein levels of MnSOD and catalase (Burgering and Medema 2003), which are the two major antioxidant enzymes responsible for

protecting organisms against oxidative stress by reducing ROS. In the present study, the levels of antioxidant enzymes during aging and CR were analyzed by

Fig. 2 Translocation of FoxO6 by oxidative stress. **a** H₂O₂ (100 μ M) time-dependently inhibited the activation of FoxO6. Western blot was used to detect FoxO6 in the cytoplasmic and nuclear extracts (20 μ g protein) of HEK293T cells. **b** H₂O₂ dose-dependently inhibited the activation of FoxO6. Western blot was used to detect FoxO6 cytoplasmic and nuclear extracts (20 μ g

protein) of HEK293T cells. **c** DCFDA fluorescence intensities were measured after treating HEK293T cells with vehicle or H₂O₂ for 1 or 6 h. Results of one-factor ANOVA: *** $p<0.001$; ** $p<0.01$; * $p<0.05$; vs. H₂O₂ untreated group, respectively. **d** Indirect immunohistochemistry findings showing the effect of FoxO6 on H₂O₂-induced subcellular localization in HEK293T cells

Fig. 3 Increased FoxO6 phosphorylation and activation during aging and their inhibition by CR. **a** Nuclear extracts were prepared from young and old rat kidneys. Immunoprecipitation showed FoxO6 was physically associated with p-FoxO6 and 14-3-3β. **b** Western blot analyses for renal nuclear p-FoxO6 and FoxO6 were

performed on nuclear proteins from AL and CR rats. The results shown are representative of three experiments. Results of one-factor ANOVA: ** $p < 0.01$ vs. young age AL rats; *** $p < 0.001$ vs. old age AL rats

Western blotting. As shown in Fig. 4, although MnSOD and catalase levels decreased during aging, MnSOD levels were similar in the AL and CR groups. On the other hand, catalase protein levels were greater in the CR group. These observations indicate the importance of the role played by FoxO6 regarding antioxidant enzyme expression during aging.

Modulation of insulin level, RS, and the PI3K/Akt signaling pathway by CR during aging

To assess overall oxidative status, total RS levels were measured in kidney tissue using a DCFDA probe, as evidence indicates that mammalian FoxO protects human cells from oxidative damage (Kops et al. 2002). In

Fig. 4 Effects of age and CR on gene expressions of MnSOD, catalase, and FoxO6-dependent genes. Effects of age and CR on the expression of catalase and MnSOD. Western blotting was used to determine catalase and MnSOD protein levels in the kidney tissues of young (6-month-old) and old (24-month-old) AL and

CR rats. The results shown are representative of three experiments. Results of one-factor ANOVA: *** $p < 0.001$ vs. Y-AL control; *** $p < 0.001$ vs. Y-CR control; ** $p < 0.01$ vs. age-matched AL rats; * $p < 0.05$ vs. age-matched CR rats. Y young, O old, AL ad libitum, CR calorie restriction

the present study, RS levels were found to be upregulated in rats and CR reduced this upregulation (Fig. 5a), indicating that CR acts to redress redox imbalance during aging.

In addition, we examined the signaling molecules that lead to Akt activation (via PI3K activation) by oxidative stress. To determine whether FoxO phosphorylation is induced by activation of the PI3K/Akt pathway, we examined phosphorylated Akt (the active form of Akt) levels. Although total Akt levels did not change, aging was found to increase Akt phosphorylation at Ser473 (Fig. 5), and CR suppressed this effect (Fig. 5b). These findings suggest that PI3K/Akt signaling, which is upregulated by increased oxidative stress, is associated with FoxO6 phosphorylation during aging and that CR inhibits these effects.

We also examined the expressions of key signals of Akt activation and insulin upregulation. Insulin levels

were observed to increase during aging (Fig. 5c), and CR markedly reduced these increases (Fig. 5c). Furthermore, it is well established that insulin activates PI3K and its downstream target, Akt. These results suggest that PI3K/Akt signaling, which is upregulated by increased insulin levels and oxidative stress, is associated with FoxO6 phosphorylation during aging and that CR inhibits these effects.

Verification of enhanced FoxO6 phosphorylation in H₂O₂-treated HEK293T cells

We examined FoxO6 expression in HEK293T cells exposed to oxidative stress, by treating FoxO6-virus-treated cells with or without different concentrations (50 to 500 μ M) of H₂O₂. As shown in Fig. 6a, treatment with 100 μ M H₂O₂ induced a remarkable shift of FoxO6 from the nucleus to the cytoplasm. Furthermore, levels of catalase were increased by

Fig. 5 Effects of age and CR on insulin signaling molecules. **a** RS generation in aged rats and the effects of aging and CR were investigated using DCFDA and kidney homogenates. Values are the mean $\pm$ SEs of five rats. Results of one-factor ANOVA: # p < 0.05 vs. young age AL rats; * p < 0.05 vs. old age AL rats. \$ p < 0.05 vs. Old AL rats. **b** CR prevented age-related activation of the PI3K/Akt, IKK, and ERK pathways. Western blotting was

used to determine total and phosphorylated Akt (Ser 473) levels in cytoplasmic extracts (20 μ g protein) from AL or CR rats. **c** Levels of insulin in the serum of young and old AL or CR rat were measured using a chemical reagent kit (n = 6 per group). The results shown are representative of three experiments. Results of one-factor ANOVA: ** p < 0.01 vs. young AL rats; * p < 0.05 vs. old AL rats

Fig. 6 Enhancement of FoxO phosphorylation in H₂O₂-treated HEK293T cells. HEK293T cells were treated with H₂O₂ at various concentrations, and FoxO6 levels were determined by Western blotting. Samples loaded on sample gels were probed with β-actin and histone H1. **a** Levels of nuclear FoxO6 and cytoplasmic catalase were noticeably diminished after treatment with H₂O₂ at 50 to 500 μM. **b** Nuclear phosphorylated FoxO6 and catalase levels were noticeably decreased by 100 μM H₂O₂ in FoxO6-

transfected (100 MOI) cells. **c** Quantitative analysis was performed by measuring DCFDA fluorescence after treating cells with vehicle or 100 μM H₂O₂ in the absence or presence of FoxO6 (100 MOI), CA-Akt (25, 100 MOI), or FoxO6-siRNA (100 MOI) for 1 day. Results of one-factor ANOVA: ***p* < 0.01 vs. H₂O₂ untreated cells; ****p* < 0.001 vs. H₂O₂-treated cells. **d** Immunoprecipitated FoxO6 was found to be physically associated with 14-3-3β by Western blotting

enhanced FoxO6 expression but reduced by adding H₂O₂ (Fig. 6b). In addition, we found that RS levels were significantly increased by H₂O₂ in the presence of empty vector and decreased by FoxO6 virus (Fig. 6c).

We also examined the effect of oxidative stress on FoxO phosphorylation. As shown in Fig. 6d, H₂O₂ enhanced FoxO6 phosphorylation at Ser184 and reduced unphosphorylated FoxO6 levels in H₂O₂-treated HEK293T cells. Phosphorylated FoxO6 is known to associate with 14-3-3β (a scaffold protein), which binds with FoxO6 and modifies its transcriptional activity (Kim et al. 2011), and thus, we generated an adenoviral vector expressing a constitutively active allele of FoxO6-CA by converting the conserved Akt phosphorylation site at Ser¹⁸⁴ to Ala¹⁸⁴ (Kim et al. 2011).

However, it was found that FoxO6-CA did not change FoxO6 phosphorylation (Supp. 1) or H₂O₂ levels.

Regulation of FoxO6 activity by oxidative stress in HEK293T cells

The transcriptional activities of the FoxO family proteins have been reported to increase when insulin levels are reduced (Barthel et al. 2005). To examine the hypothesis that FoxO6 targets the catalase gene for transactivation, we examined the ability of FoxO6 to stimulate catalase expression in HEK293T cells. FoxO6 was shown to be associated with binding and activity to conserve H₂O₂ in the catalase promoter, as determined by luciferase (Fig. 7a) and the chromatin

Fig. 7 Activation of FoxO6 by oxidative stress in kidney cells. **a** HEK293T cells were transiently transfected with a catalase-containing plasmid linked to the luciferase gene, preincubated with FoxO6 (100 MOI) or FoxO6-siRNA (100 MOI) for 8 h, and then treated with H₂O₂ for 2 h. Results are presented in relative luminescence units (RLU). Results of one-factor ANOVA: ****p*<0.001 vs. untreated control group, #*p*<0.05; ##*p*<0.01; ###*p*<0.001 vs. reporter-only-

transfected group. **b** FoxO6 bound to catalase promoter in HEK293T cells. Cells were transfected with catalase-Luc in the presence of FoxO6 vector at 100 MOI. After incubation for 24 h, cells were subjected to chromatin immunoprecipitation (ChIP) assay using rabbit pre-immune IgG (lanes 1 and 2), anti-FoxO6 antibody (lanes 3 and 4), and 100 μM H₂O₂ (lanes 5 and 6). Immunoprecipitates were subjected to PCR using the catalase promoter

immunoprecipitation assay (Fig. 7b) in the empty vector and the FoxO6 virus-transduced HEK293T cells. FoxO6 associated with the catalase promoter DNA by FoxO6 virus cells, compared with the empty vector in HEK293T cells, enhanced the stimulatory effect of FoxO6 on catalase expression. This action was abolished in response to H₂O₂ in kidney cells (Fig. 7b). On the other hand, catalase promoter activity was reduced in FoxO6-siRNA-treated cells (Fig. 7a). It also reduced catalase levels in FoxO6-siRNA vector-transduced cells (Supp. 2). These observations support the idea that FoxO6 targets the catalase gene for transactivation and, thus, contributes to the regulation of oxidative stress.

Effect of oxidative stress on FoxO6 phosphorylation and the PI3K/Akt pathway

Several authors have suggested that members of the FoxO family are regulated by the PI3K/Akt pathway. More specifically, Akt (a key downstream effector of PI3K) is believed to phosphorylate FoxOs directly or to promote their phosphorylation by other kinases (Brunet

et al. 1999). We examined the PI3K/Akt pathway and its effect on FoxO6 phosphorylation utilizing constitutively active Akt (CA-Akt) and measured FoxO6 phosphorylation by Akt. We found that FoxO6 reduction due to its phosphorylation increased when the concentration of Akt was increased (Fig. 8a).

The nuclear and cytoplasmic levels of p-Akt have been reported in 97 and 100 %, respectively, of astrocytomas (El-Habr et al. 2010), and Chang et al. (2011) reported that Akt activity was suppressed by the dephosphorylation of Akt. Accordingly, we treated HEK293T cells with FoxO6 or CA-Akt (25 MOI) for 1 day, incubated them with 100 μM H₂O₂ for 1 h, and then assessed p-FoxO6 and p-Akt levels. As shown in Fig. 8b, in HEK293T cells, FoxO6 remained predominantly in the nucleus regardless of CA-Akt. These results indicate that the phosphorylation of FoxO6 in kidney cells is associated with the phosphorylation and nuclear accumulation of Akt.

Furthermore, immunostaining showed that FoxO6, although phosphorylated in response to H₂O₂, was localized in the cytoplasm in H₂O₂-treated HEK293T cells, as determined by immunostaining (Fig. 8c);

Fig. 8 Activation of FoxO6 phosphorylation through the PI3K/Akt pathway by H₂O₂. HEK293T cells were grown to 80 % confluence in 100 mm dishes in DMEM medium, pretreated (1 day) with or without FoxO6 (100 MOI) plus CA-Akt (25 MOI), and then stimulated with 100 μ M H₂O₂. **a** Cells were pretransduced with 100 MOI of FoxO6 vector in the absence or presence of Ca-Akt vector (10–200 MOI) and analyzed by Western blotting using antibody. **b** After stimulation with H₂O₂ (50 or

100 μ M) in the absence or presence of FoxO6 plus CA-Akt (25 MOI), levels of phosphorylated FoxO6 were determined in the cell extract. **c** HEK293T cells were pretransduced with 100 MOI of FoxO6 vector with or without Ca-Akt vector (25 MOI) for 24 h and then treated with or without H₂O₂ (100 μ M) for 1 h. Cells were immunostained using a rabbit anti-FoxO6 antibody, followed by IgG conjugated with fluorescein isothiocyanate (green). Bar=100 μ m

otherwise, FoxO6 did not undergo a low (25 MOI) Akt-dependent nuclear exclusion. These findings indicate that H₂O₂ phosphorylates FoxO6 in an Akt-dependent manner and that this results in its cytoplasmic translocation.

Discussion

In the present study, we provide new evidence that the FoxO6 phosphorylation that occurs during aging can be prevented by CR. Our study also shows that serum insulin levels and ROS increase with age and that CR suppresses these increases. Changes in insulin level and its modulation are deemed significant because increased

FoxO6 phosphorylation during aging is blunted by activated PI3K/Akt, the level of which is insulin dependent. As was expected, the insulin-suppressive action of CR prevented age-associated FoxO6 decrease by inhibiting the PI3K/Akt pathway (Fig. 5). Zemva et al. (2012) reported that FoxO6 mRNA levels are upregulated in aged mouse brain, which implies that the insulin/FoxO6 signaling pathway is involved in aging. However, clinical studies have failed to reveal any association between FoxO6 and life expectancy in human (Kleindorp et al. 2011). The most significant finding of this study is that decreased FoxO binding activity during aging is blunted by PI3K/Akt activation during aging under states of increased insulin and oxidative stress. As one might expect, our data on CR shown in Fig. 1

suggest that the oxidative stress-suppressive effect of CR prevents FoxO6 reduction by inhibiting the PI3K/Akt pathway.

The protective role of FoxO during aging has been suggested by several authors; for example, in one study, the loss of FoxO3 activity in explanted vascular smooth muscle of aged animals appeared to limit antioxidant properties in tissues by downregulating MnSOD and enhancing cell injury (Li et al. 2006). Yamaza et al. (2010) reported that FoxO1 also regulates some genes involved in cell cycle arrest, DNA repair, apoptosis, and stress reactions in response to oxidative stress in the liver. Nuclear FoxO1 activates the transcriptions of genes involved in stress response, including MnSOD, to counteract the effect of enhanced mitochondrial ROS production (Senapedis et al. 2011). Furthermore, the FoxO1-mediated transcriptions of antioxidant genes, such as MnSOD, are facilitated by the activation of FoxO1 via its deacetylation by Sirt1 (Jian et al. 2011). However, no previous study has investigated the role of FoxO6 during aging.

Initial clues that PI3K controls FoxO activity resulted from studies on the nematode, *Caenorhabditis elegans*. Genetic studies showed that PI3K suppresses the function of DAF-16 (a Forkhead transcription factor) (Lin et al. 1997), and it was subsequently found that PI3K is critical for the proper control of metabolism and cell survival (Birkenkamp and Coffey 2003). However, age-related interactions between PI3K and FoxO6 activities have not been fully explored (Zemva et al. 2012).

The aging process and ad libitum feeding of animals are well known to increase insulin and IGF-1 plasma levels (Coschigano et al. 2003). In response to insulin, FoxO proteins are phosphorylated by PKB, a downstream kinase of PI3K, and this phosphorylation leads to the translocation of these proteins from the nucleus to the cytoplasm (Brunet et al. 1999). The inhibition of FoxO and other related proteins through phosphorylation by Akt also has been reported (Tang et al. 1999). Moreover, Lin et al. (2004) reported that FoxO3a may have similar immunoregulatory functions in vivo. Protein kinase B (PKB/c-Akt) mediates many of the anti-apoptotic effects of PI3K signaling. However, although a large number of PKB substrates have been implicated in the regulation of cellular survival, little is known as to how PI3K/Akt signaling regulates cellular ROS levels. Earlier investigations have shown that Akt-regulated FoxO can reduce cellular oxidative stress by

directly increasing mRNA and protein levels of MnSOD and catalase (Burgering and Medema 2003). In the present study, a similar situation was observed whereby Akt activation decreased catalase levels and, thus, probably increased cellular ROS.

Our results characterize FoxO6 as a novel transcription factor that plays an important function in the aged kidney, as was demonstrated by the modulation of FoxO6 by insulin signaling in cultured HEK293T cells. Furthermore, we found that the PI3K/Akt pathway was activated, FoxO6 was phosphorylated (Ser184), and target gene expressions were downregulated when these cells were exposed to H₂O₂ (Fig. 6). It is known that FoxO1 is regulated by cyclin-dependent kinases (CDK1/2) (Yuan et al. 2008); FoxO3 is phosphorylated by Ste20-like kinase (MST1) (Lehtinen et al. 2006); and FoxO4 is phosphorylated by stress-activated protein kinase (JNK), and that all of these processes result in relocalization from the nucleus to the cytoplasm (Oh et al. 2005). Kim et al. (2011) found that in liver cells, FoxO6 is mainly located in the nucleus in serum-containing media and that the nuclear localization of FoxO6 implies transcriptional activity under these conditions. On the other hand, FoxO6 was found not to be regulated by nucleocytoplasmic shuttling (Jacobs et al. 2003). Our observation that the cytosolic level of FoxO6 was slightly increased by H₂O₂ indicates the ability of FoxO6 to translocate; however, we also found that the FoxO6 protein was predominantly localized in the nucleus (Fig. 8c).

The present study provides additional information on FoxO6 transcriptional activity and reveals the phosphorylation of PI3K/Akt during aging. Furthermore, it suggests that these changes are closely related to age-related increases in insulin and oxidative stress levels. More importantly, in the context of aging, these age-related changes were found to be counterbalanced by the anti-aging effect of CR. Based on our in vivo and in vitro observations, we propose that the age-related phosphorylation of FoxO6 represses the expressions of catalase and MnSOD. Furthermore, we suggest that FoxO6 are regulated by PI3K/Akt activation induced by age-related oxidative stress, which in turn can be modulated by the anti-aging effect of CR.

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Conflict of interest The authors have no conflict of interest to disclose.

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