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RESEARCH ARTICLE

The activation of NF-κB through Akt-induced FOXO1 phosphorylation during aging and its modulation by calorie restriction

Dae Hyun Kim · Ji Young Kim · Byung Pal Yu · Hae Young Chung

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Abstract Insulin-induced PI3K/Akt activation is known to inhibit a family of Forkhead transcription factors (FOXO), which can lead to increased oxidative stress in several model organisms. One of major transcription factors activated by oxidative stress and responsible for the production of many proinflammatory cytokines is NF-κB. In the present study, We were carried out to determine the relationship between FOXO1 and NF-κB activation using HEK293T cells and aged kidney isolated from ad libitum fed (AL) and 40% calorie restriction (CR) rats. Results showed that phosphorylation of FOXO1 and NF-κB activation were significantly increased in old rats. Moreover, FOXO1 phosphorylation and NF-κB activation were shown to be significantly lower in the CR rats compared with 24–month-old AL rats. To further explore the molecular link between FOXO and NF-κB, we performed transfection experiments with FOXO-mutant plasmid in cultured HEK293T cells. Treatment of the cell with insulin led to NF-κB activation through the phosphorylation of FOXO via the PI3K/Akt pathway. These results indicate that insulin promoted NF-κB activation through phosphorylation of FOXO1 by upregulating PI3K/Akt signaling. We conclude that the phosphorylation of FOXO1 regulates NF-κB nuclear translocation by activating PI3K/Akt during aging, which was suppressed by the hypoinsulinemic action of CR.

Keywords Insulin · FOXO1 · NF-κB · PI3K/Akt pathway · Calorie restriction · Aging

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Introduction

The FOXO (Forkhead transcription factor) family in mammals consists of the evolutionally highly conserved forkhead transcription factors, FOXO1, FOXO3a, FOXO4, and FOXO6 (Van der Heide et al. 2004). A recent series of investigations have demonstrated that FOXO factors play key roles in inducing various downstream target genes, including the regulators of metabolism, cell cycle, cell death, and oxidative stress response (Accili and Arden 2004).

One of the key regulatory mechanisms of FOXO is the phosphorylation step. In response to insulin or

growth factors, for instance, FOXO proteins are phosphorylated by protein kinase B (PKB, also known as Akt), a downstream kinase of phosphatidylinositol 3-kinase (PI3K), which results in the translocation of FOXO from the nucleus into the cytoplasm (Brunet et al. 1999).

More intricate interactions between Akt and FOXO in the cellular regulatory mechanisms have been recently revealed. For instance, in yeast, the mutation of Sch 9, which is homologous to Akt, extends lifespan (Fabrizio et al. 2001) and the mutation of the insulin receptor that decreases activity in the insulin/IGF-1-like pathway increases the longevity of fruit flies (Tatar et al. 2001) and mice (Bluher et al. 2003). It is interesting to note 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). Recently, others have shown that the PKB-regulated FOXO can reduce levels of cellular oxidative stress by directly increasing mRNA and protein levels of manganese superoxide dismutase (MnSOD) and catalase (Burgering and Medema 2003). However, at present, little information is available on the status of FOXO during aging, and how it is modulated by various signaling factors and the anti-oxidative, anti-aging action of calorie restriction (CR).

Redox-responsive nuclear factor-kappa B (NF-κB) controls the expression of various gene products that affect important cellular processes, such as inflammation, adhesion molecules, cell cycle, angiogenesis, and apoptosis (Kim et al. 2000). Transcriptional active NF-κB is typically composed of a heterodimareric protein complex that contains a DNA-binding component and an acidic transactivation domain. The best-studied and most prevalent form of NF-κB exists as a heterodimer composed of p50 and RelA/p65 polypeptides. Under normal conditions, NF-κB resides in the cytoplasm bound by its inhibitory proteins (Baldwin 1996), which are known to be modulated by aging and CR (Kim et al. 2000).

Several recent studies have explored age effects on the regulation of the redox sensitive transcription factor NF-κB. Consistent findings show activated NF-κB activity in heart, liver, kidney, and brain tissues from old rodents (Kim et al. 2002). The age-related activation of NF-κB activity has been linked to increased oxidative stress during aging, which was shown to be effectively suppressed by CR (Kim et al. 2000). These results provide strong experimental evidence supporting the notion that increased NF-κB activity in aged animals is likely due to a shift in intracellular redox balance during aging. However, there is still an unsettled question on whether Akt activates NF-κB through IκB (Ozes et al. 1999), or through phosphorylation of the catalytic p65 subunit of NF-κB (Sizemore et al. 1999).

In recent years, studies focused on the role of insulin in signaling and on the manner in which abnormalities of insulin metabolism contribute to physiological disorders in aging (Craft 2005). It was reported that insulin modulates complicated aspects of the inflammatory network, with anti-inflammatory actions observed at low doses of insulin and pro-inflammatory effects at high levels insulin (Dandona et al. 2002).

CR has been shown to delay age-related biologic changes and suppress a number of age-associated pathologic abnormalities in both genders and across mammalian and non-mammalian species (Yu 2005). One consistent finding in CR animals is reduced oxidative stress, providing a well-maintained redox status (Yu 2005), which greatly impacts the regulation of many redox-sensitive transcriptomes and their gene expressions.

In the present study, we investigated how PI3K/Akt signaling modulates FOXO and NF-κB activities with aging, utilizing HEK293T cells and aged kidney isolated from ad libitum fed (AL) and 40% CR rats.

Materials and methods

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). The radionucleotide [γ-^{32}P]-ATP (250 μCi), and Western blotting detection reagents were obtained from Amersham (Bucks, UK). RNAzol^{TM} B was obtained from TEL-TEST, Inc. (Friendwood, TX, USA). Antibodies against FOXO1, p-FOXO1 (Thr24), catalase, MnSOD, p65, p50, IκBα, β-actin, Histone H1, PI3K (p85α), 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, Hertsfordshire, UK). Anti-rabbit IgG-horseradish peroxidase-conjugated antibody and anti-mouse IgG-horseradish peroxidase-conjugated antibody were obtained 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).

Animals

Specific pathogen-free male Fischer 344 rats (6 and 24 months of age) 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% α-methionnine, 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 food, beginning at 6 weeks of age.

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 phenylmethyl-sulfonylfluoride (PMSF), 1 μM pepstatin, 2 μM sodium orthovanadate (pH 7.4)]. The tissue was immediately frozen in liquid nitrogen and stored at −80°C. Histopathological examination revealed no evidence of nephritic lesions detected in these soy-protein fed rats even at 24 months of age (Iwasaki et al. 1988). The selection of kidney was made because the kidney is metabolically active, sensitive to many ages-relate changes including, redox responsive molecular events making the kidney suitable for the study. In addition, our lab has substantial experience and data on age-related renal changes.

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), 233.6 mg/ml glutamine, 100 mg/ml penicillin streptomycin, and 0.25 μg/ml amphotericin B, and 10% heat-inactivated fetal bovine serum. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Cells were serum-starved for a further 24 h prior to serum treatment. For exposure to serum, cells were washing twice with pre-warmed PBS and incubated in the presence of serum-free medium.

Plasmid

For the luciferase assay, pNF-kB-Luc vector was purchased form Clontech (Clontech, CA, USA). Expression plasmid pcDNA FKHR and FKHR AAA (T24A, S256A, S319A) were purchased from Addgene (Addgene Inc, USA).

Nuclear extract preparation

The frozen rat kidney tissue (0.2–0.4 g) was rinsed in PBS buffer and then transferred to the 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 DTT, 0.5 mM PMSF) was added as the 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).

Western blotting

Western blotting was carried out as described previously (Habib et al. 1993). 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% bromphenol blue) at a ratio of 1:1. Total protein-equivalents for each sample were separated by SDS-PAGE using acrylamide gels as described by Laemmli (1970), and transferred to PVDF membrane at 15 V for 1 h in a semi-dry transfer system. The membrane was immediately placed into a blocking buffer (1% non-fat 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. Pre-stained protein markers were used for molecular weight determinations.

Transfection and luciferase reporter assay

The activity of NF-κB was examined using the luciferase plasmid DNA, pTAL-NFκB that contains a specific binding sequence for NF-κB (BD Biosciences Clontech, Mountain View, CA, USA). Transfection was carried out using FuGENE 6 Reagent (Roche, Indianapolis, IN). Briefly, 1.5×10^4 cells per well were seeded in 48-well plates. When cultured cells reached about 40% confluence, cells were treated with 0.1 μg DNA/0.5 μl FuGENE 6 complexes in 500 μl normal media (10% serum contained) for 42 h. Subsequently, 0.2 μM of insulin was treated after exchange to serum-free media and treatments with PI3K/Akt inhibitor (LY294002) and catalase inhibitor (3-amino-1,2,4-triazole) were performed 1 h. After an additional incubation for 6 h, cells were washed with PBS and subjected to Steady-Glo Luciferase Assay System (Promega, Medison, WI, USA). Luciferase activity was measured by a luminometer (GENious, TECAN, Salzburg, Austria).

Assay for insulin

Insulin level was determined using the rat insulin ELISA kit (Shibayagi Co, Japan). One hundred μl of biotin-conjugated anti-insulin solution was added to 10 μl of sample in each well. Chromogenic substrate solution was added to the well after addition of HRP-conjugated avidin solution to the 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).

Electrophoretic mobility shift assay (EMSA)

The EMSA method was used to characterize the binding activities of FOXO and NF-κB in nuclear extracts (Kerr 1995). Rat NF-κB oligonucleotide: 5′-GAG AGG CAA GGG GAT TCC CTT AGT TAG GA-3′. Human NF-κB oligonucleotide: 5′-AAG GAG TTG AGG GGA CTT TCC CAG GC-3′. Rat FOXO oligonucleotide: 5′-TTA GTC ATT TTG TTT GTT CAT A-3′. Human FOXO oligonucleotide: 5′-CAG GCT GTT TTT GTG TGC CTG TTT TTC TAT TTT-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 contain 5% glycerol, 1 mM $MgCl_2$, 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 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.

ROS generation

For the determination of intracellular ROS 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 LY294002, 3-amino-1,2,4-triazole and were pre-incubated for 1 h. After treatment with

insulin (100 nM) for 30 min, the medium was replaced with 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.

Statistical analysis

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

Results

Effects of insulin level and insulin signaling

The binding of insulin and growth factors to specific receptor tyrosine kinases activates PI3K and 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 (Brunet et al. 1999).

We therefore analyzed the expression of key signaling molecules leading to Akt activation and to increased insulin levels. Insulin levels increased during aging (Fig. 1a), while CR markedly reduced insulin levels (Fig. 1a). Insulin expresses its action, activating PI3K and its downstream target, Akt. Therefore, to determine whether a change in FOXO phosphorylation is caused by activation of the PI3K/Akt pathway, phosphorylated Akt (the active form of Akt) was investigated. Although the total Akt amount did not change, aging increased phosphorylated Akt in Ser473 (Fig. 1b). Conversely, CR was observed to suppress the age-related increase of phosphorylated Akt (Fig. 1b).

These data suggest that PI3K/Akt signaling, upregulated by increased insulin levels might be associated with FOXO phosphorylation during the aging process, while CR reversed these phenomena.

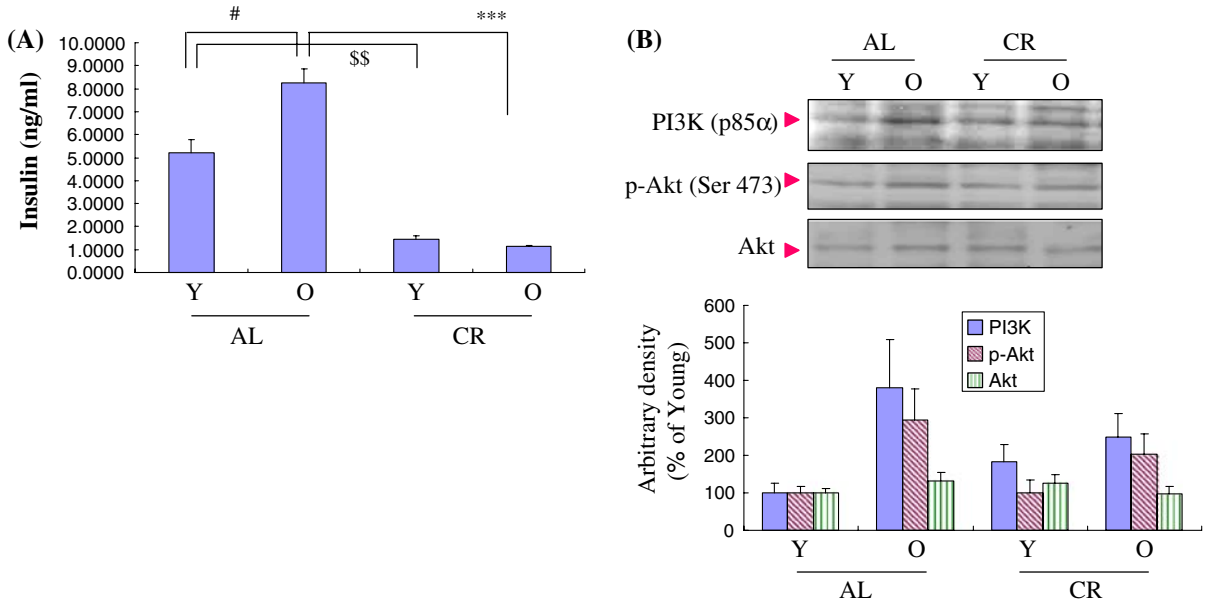

Fig. 1 Effects of age and CR in insulin level and signaling. (**a**) Levels of insulin in young and old, AL and CR rat serum were measured by chemical reagent kit ($n = 6$ in each group). (**b**) CR prevented age-related activation of PI3K/Akt pathway. Western blot analysis was performed to detect PI3K and phosphorylated Akt (Ser 473) cytoplasmic extracts (60 μg protein) from AL or CR rats, ages 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: $^{\#}P < 0.05$ vs. Young group of AL rats; $^{\$\$}P < 0.01$ vs. Young group of AL rats; $^{***}P < 0.001$ vs. age-matched AL rats. Y, young (6 month); O, old (24 month); AL, ad libitum; CR, calorie restriction

Effects of age and CR on FOXO1 phosphorylation and NF-κB activation

One of well-known, age-related changes is the increased insulin and IGF-1, which also is known to be prevented by CR (Tomita et al. 2001). At the transcriptional level, the FOXO families of proteins are increased by the reduction of insulin or IGF-1 levels (Brunet et al. 1999).

Results showed that FOXO1 phosphorylation levels were higher in old rat compared to young rats and that CR animals showed lower FOXO1 levels compared to AL animals of the same age (Fig. 2a). 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 AL rats, while little change was detected in the CR rats, even in senescent, 24-month-old rats (Fig. 2b). Binding specificity of the FOXO complex was demonstrated using a 100-fold excess of an unlabeled oligonucleotide, which competed for binding (Fig. 2b, Lane 6). Recently, others have shown that the Akt-regulated FOXO1 can reduce the level of cellular oxidative

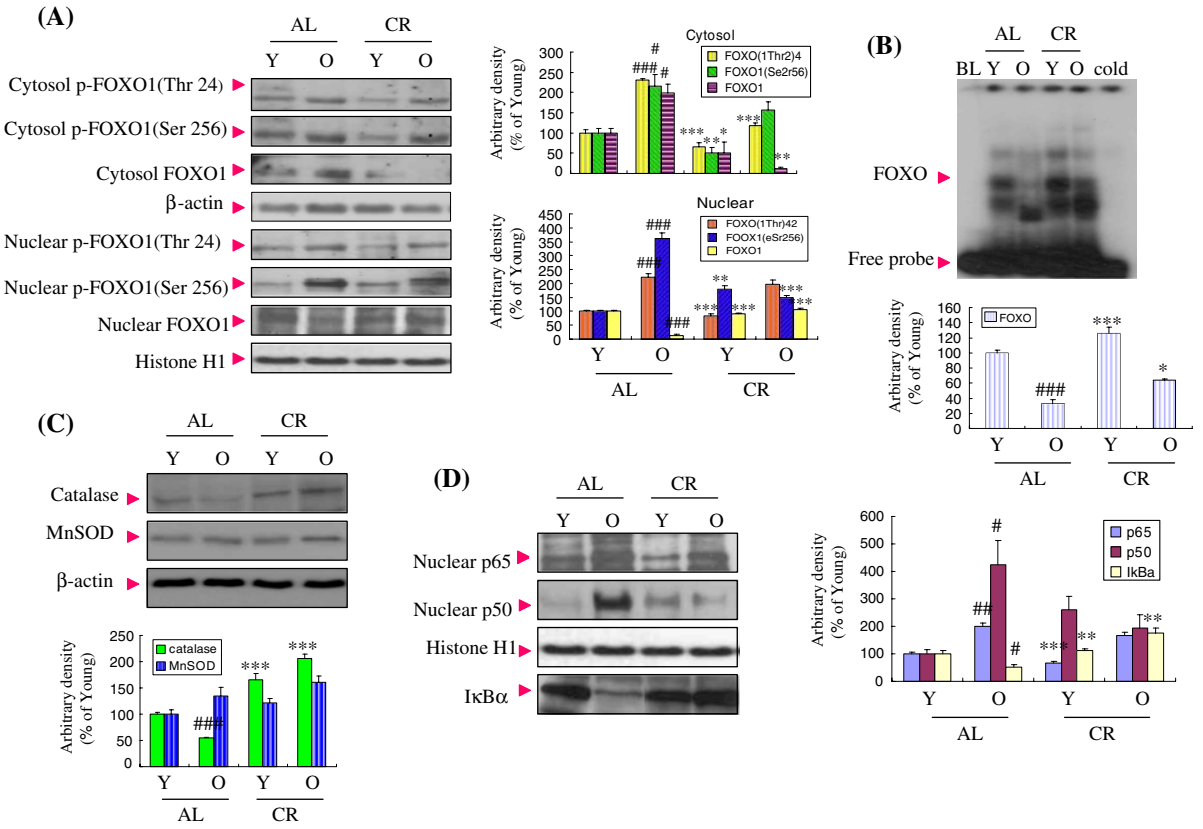

Fig. 2 Increased FOXO phosphorylation and NF-κB activation during aging, and their inhibition by CR. Western blot analyses of renal nuclear and cytosol (**a**) FOXO1 were performed from nuclear and cytosol protein of AL and CR rats. A representative result is presented from three experiments. (**b**) CR increased age-related suppression of FOXO binding activity. The EMSA method was used to compare nuclear FOXO binding activities in kidney nuclear protein from AL and CR rats. Lane 1: probe without nuclear protein sample (BL); Lane 2–5: kidney nuclear protein samples of AL and CR rats, ages 6 and 24 months. Lane 6: competition assay using a 100-fold excess of unlabeled FHRE oligonucleotide. A representative result is presented from three experiments. (**c**) Effect of age and CR on Catalase and MnSOD expression. Western blot analyses were performed to detect Catalase and MnSOD protein levels from AL and CR rats, at ages 6 and 24 months. (**d**) The suppressive action of CR on the age-related NF-κB activation. Western blotting was performed to detect nuclear localization of p50 and p65 in AL and CR rats. One representative blot of each protein is shown from three experiments that yielded similar results, respectively. Results of one factor ANOVA: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. Y-AL control; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. age-matched AL rats. Y, Young; O, Old; AL, ad libitum; CR, calorie restriction

stress by directly increasing protein levels of MnSOD and catalase (Burgering and Medema 2003).

MnSOD and catalase are two major antioxidant enzymes that play a central role in the protection against oxidative stress by reducing ROS. In these experiments, protein levels of antioxidant enzymes during aging and CR were monitored by Western blot. As shown in Fig. 2c, MnSOD levels were unchanged during the aging process, but catalase levels and activity decreased during the aging process. On the other hand, catalase gene expression and activity increased in the CR group compared to the AL group (Fig. 2c).

An assessment of age-related changes in NF-κB was performed by Western blot analysis using nuclear extracts. Results showed that the protein levels of NF-κB compounds, p65/p50 increased with age in control rats, while low protein levels of NF-κB were maintained in the CR rats. According to this result, the nuclear translocation of NF-κB also increased with age, but was prevented by CR and was regulated through the alteration of IκB (Fig. 2d). These data indicated a relationship between FOXO1 phosphorylation and NF-κB activity during the aging process.

Verification of enhanced FOXO1 phosphorylation and NF-κB activation in insulin-treated cultured cells

We examined the relationship of FOXO1 and NF-κB via the insulin signaling pathway in HEK293T cells. Cells were serum-starved overnight and then treated with or without 100 nM insulin in serum-free media from 10 to 120 min. FOXO1 and NF-κB levels were analyzed by Western blotting. As shown in Fig. 3a, treatment with 100 nM insulin induced a remarkable shift of FOXO1 from the nucleus into the cytoplasm. Nuclear FOXO1 was noticeably decreased, while its cytoplasmic counterpart markedly increased by as early as 30 min, indicating that insulin enhanced the phosphorylation of FOXO1 on Ser 256 and Thr 24. To identify the possible underlying molecular events of insulin-induced NF-κB activation in HEK293T cells, Western bolt analyses were performed to examine the nuclear translocation of NF-κB components p65 and p50. As shown in Fig. 3b, p65 and p50 were translocated from cytosol into nuclear in response to the insulin challenge.

Effect of insulin on FOXO1 phosphorylation, NF-κB activation and PI3K/Akt

Several studies suggest that the FOXOs are regulated by the PI3K/Akt pathway. Specifically, Akt, a key downstream effector of PI3K, is thought to either phosphorylate FOXO directly (Brunet et al. 1999; Tang et al. 1999), or to promote their phosphorylation by other kinases (Brunet et al. 1999). We examined the PI3K/Akt pathway and its effect on FOXO1 phosphorylation utilizing the PI3K/Akt inhibitor, LY294002.

For these experimentations, serum-starved HEK293T cells were treated with 10 μM LY294002 for 1 h prior to a 30 min treatment with 100 nM insulin. As show in Fig. 4a, insulin enhanced the FOXO1 phosphorylation on Ser 256 and Thr 24, a site targeted by Akt. Since we observed the induction of NF-κB activation by insulin, tests were carried out to examine the ability to block NF-κB activation through Akt signaling and catalase using specific inhibitors of Akt (LY294002) and catalase (3-amino-1,2,4-triazole). Results showed that the level of NF-κB was reduced in insulin-induced HEK293T cells by the Akt inhibitor and was increased by the catalase inhibitor (Fig. 4b). To further validate the finding of LY294002, we performed transient transfection of pTAL-NF-κB to HEK293T cells. The luciferase activity was increased after insulin treatment in the transfected cells, and LY294002 suppressed this luciferase activity (Fig. 4c).

Evidence for insulin enhancement of Akt phosphorylation that is blocked by 10 μM LY294002 is shown in Fig. 4d. Results indicated that insulin upregulated FOXO1 phosphorylation and NF-κB activation by PI3K-dependent Akt phosphorylation in HEK293T cells, leading to NF-κB activation.

To determine the extent of insulin-related modulation of FOXO, EMSA was carried out with nuclear proteins. Results showed insulin-induced decreases in the nuclear binding activity of FOXO in HEK293T cells, while LY294002, the Akt inhibitor, increased FOXO binding activity (Fig. 4e). The binding specificity of the FOXO complex was demonstrated by using a 100-fold excess of an unlabeled oligonucleotide, which competed for the binding (Fig. 4e, Lane 5). Data showed insulin-induced increases in the nuclear binding activity of NF-κB in HEK293T cells, but that the Akt inhibitor, LY294002 decreased NF-κB

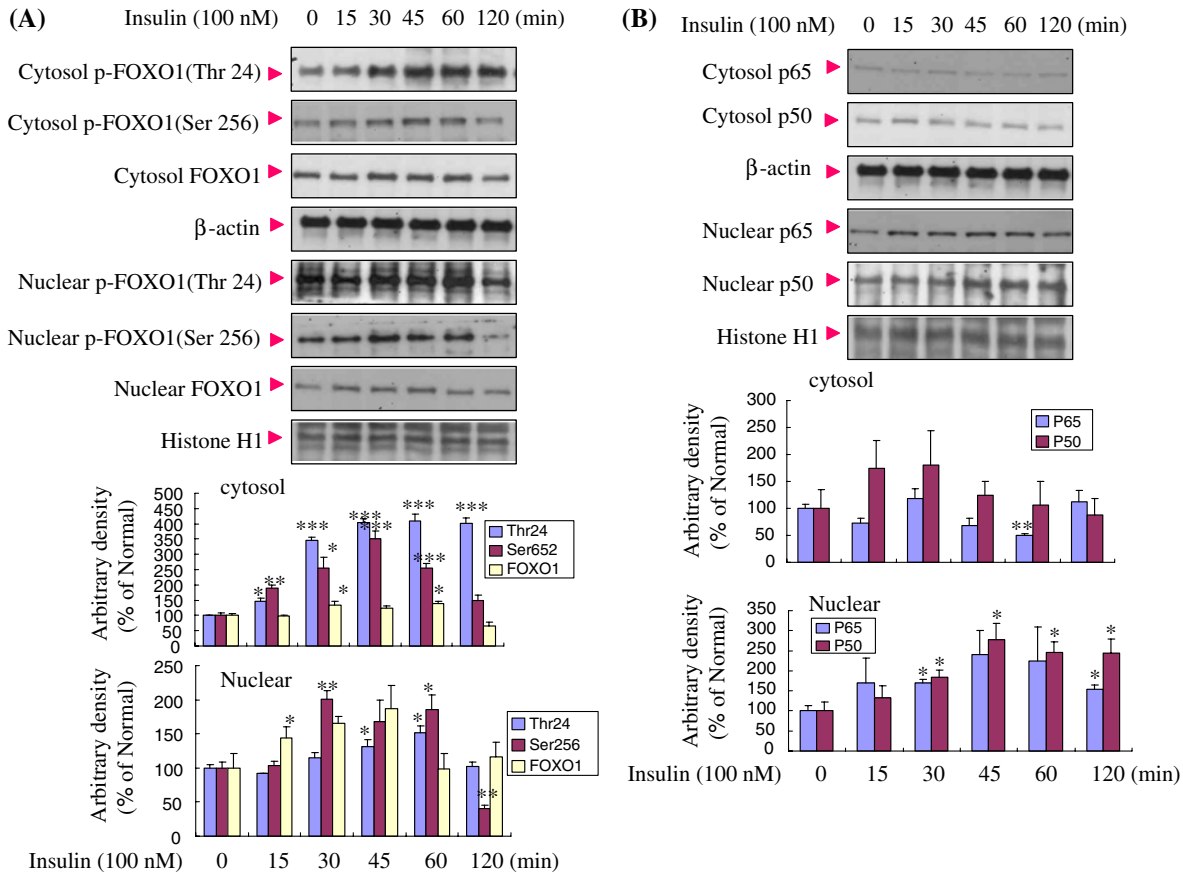

Fig. 3 Enhancement of FOXO phosphorylation and NF-κB expression in insulin-treated HEK293T cells. HEK293T cells were serum starved and treated with 100 nM of insulin for different time periods. The FOXO1 and NF-κB levels were determined by Western blotting. Samples loaded on the sample gel were probed with β-actin and Histone H1. (**a**) Nuclear and cytoplasmic levels of FOXO1 phosphorylation were noticeably increased by as early as 30 min post-treatment. (**b**) Nuclear levels of NF-κB expression were noticeably increased by as early as 30 min post-treatment, while cytoplasmic NF-κB levels were markedly decreased. Results of one factor ANOVA: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. insulin untreated group

binding activity and the catalase inhibitor increased NF-κB binding activity (Fig. 4f). The NF-κB complex binding specificity was demonstrated using a 100-fold excess of an unlabeled oligonucleotide (Fig. 4f, Lane 6).

Effects of insulin treatment on oxidative stress and gene expression of catalase and COX-2 in cultured cells

Recent evidence indicates that mammalian FOXO upregulates the free radical scavenger genes MnSOD and catalase, which have a protective effect against oxidative damage in human cells (Kops et al. 2002). To investigate the role of FOXO1 in insulin-induced oxidative stress, we examined the expressions of the antioxidant, catalase and proinflammatory COX-2. Similar to findings using the catalase inhibitor, the HEK293T cell with insulin treatment showed an increase in ROS, while the Akt inhibitor (LY294002) suppressed ROS production in these cells (Fig. 5a). Also, the levels of catalase, but not MnSOD, were reduced in HEK293T cells by insulin treatment (Fig. 5b). These results strongly suggest that constitutive activation of Akt increased the phosphorylation of FOXO, thereby downregulating catalase, leading increased ROS that promoted oxidative stress-induced inflammation via the NF-κB pathway.

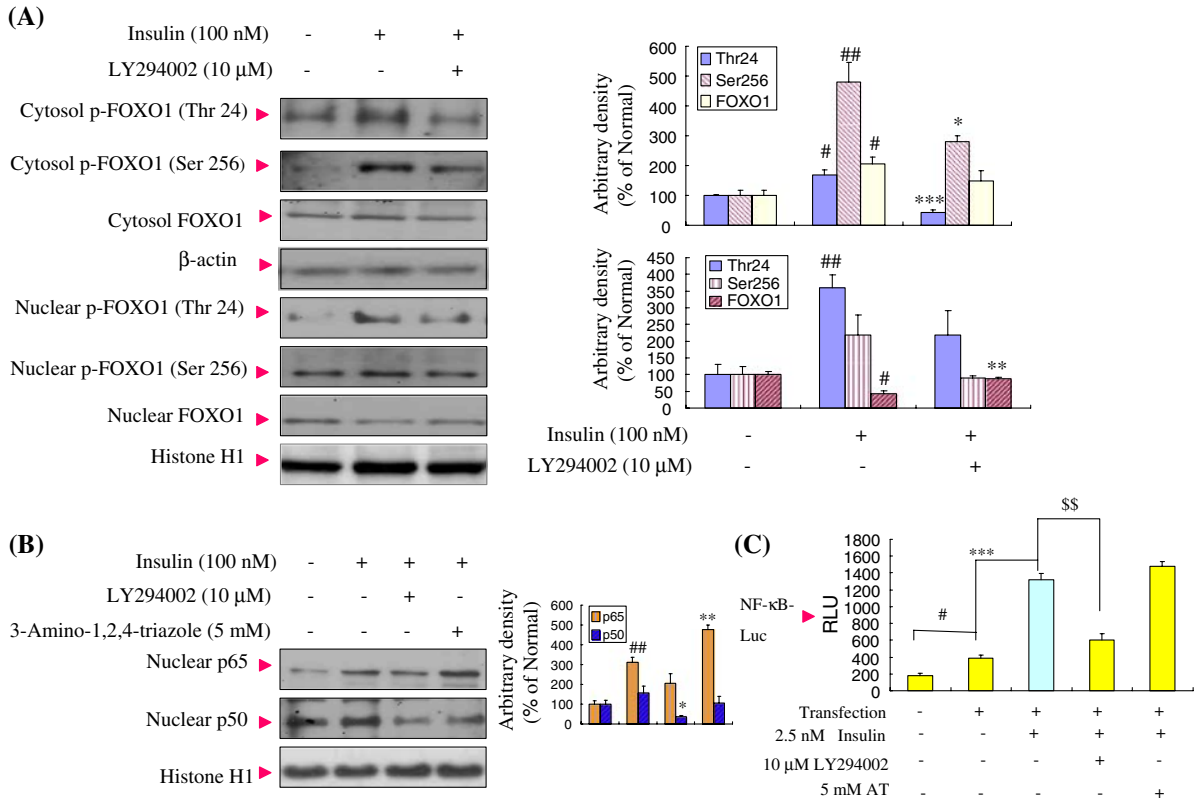

Fig. 4 Regulation of FOXO phosphorylation and NF-κB activation through PI3K/Akt pathway by insulin. HEK293T cells were grown to 80% confluence in 100 mm dishes in DMEM medium. Cells were pre-treated (1 h) with inhibitors, and then stimulated with 100 nM insulin. (**a**) After stimulation with insulin in the absence (−) or presence (+) of PI3K/Akt inhibitor (LY294002, 10 μM), FOXO1 phosphorylation was determined from the cell extract. (**b**) After stimulation with insulin (1 h for NF-κB) in the absence (−) or presence (+) of LY294002 (10 μM) and catalase inhibitor (3-amino-1,2–4-triazole, 5 mM), the cells lysed and were extracted from nucleus and cytoplasmic. The Western blot was performed for p50 and p65. (**c**) Cells were transiently transfected with an NF-κB-containing plasmid linked to the luciferase gene, and then cells were co-treated with insulin for 4 h after pre-incubation with PI3K/Akt inhibitor (LY294002, 10 μM) and catalase inhibitor (3-amino-1,2,4-triazole, 5 mM) for 1 h. Results are represented as relative luminescence unit (RLU). Statistical significance of differences between untreated control and treated groups in NF-κB-dependent luciferase activity was determined using one-way analysis of variance (ANOVA): $^{\#}P < 0.05$ vs. vector control, $^{***}P < 0.001$ vs. untreated insulin group, $^{\$\$}P < 0.01$ vs. treated insulin group, respectively. (**d**) After stimulation with insulin in the absence (−) or presence (+) of LY294002 (10 μM), the cells were lysed and determined as PI3K, p-Akt, and total-Akt. One representative experimental blot of each protein is shown from three experiments that yielded similar results, respectively. (**e**) The EMSA method was used to compare nuclear FOXO binding activities in HEK293T cells. The cells were incubated in serum-free medium with insulin (100 nM) for 1 h after pretreatment for 1 h with LY294002 (10 μM). (**f**) The cells were incubated in serum-free medium with 100 nM insulin for 1 h after pretreatment for 1 h with LY294002 (10 μM) and 3-amino-1,2,4-triazole (5 mM). Cell lysates were harvested and subjected to EMSA for DNA-binding activity. Results of one factor ANOVA: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. insulin untreated group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. insulin treated group, respectively

Role of FOXO phosphorylation in insulin-induced NF-κB activation

To investigate the interaction of NF-κB and FOXO1, HEK293T cells were incubated with insulin in the presence or absence of FOXO1 wild or mutant type. Incubation with insulin was shown to induce NF-κB, whereas the insulin-induced increase of NF-κB was blocked by transduction with mutant-FOXO1. Transduction with NF-κB, which can be partially inactivated by FOXO1 signaling, increased the insulin-stimulated wild-FOXO1 (Fig. 6a).

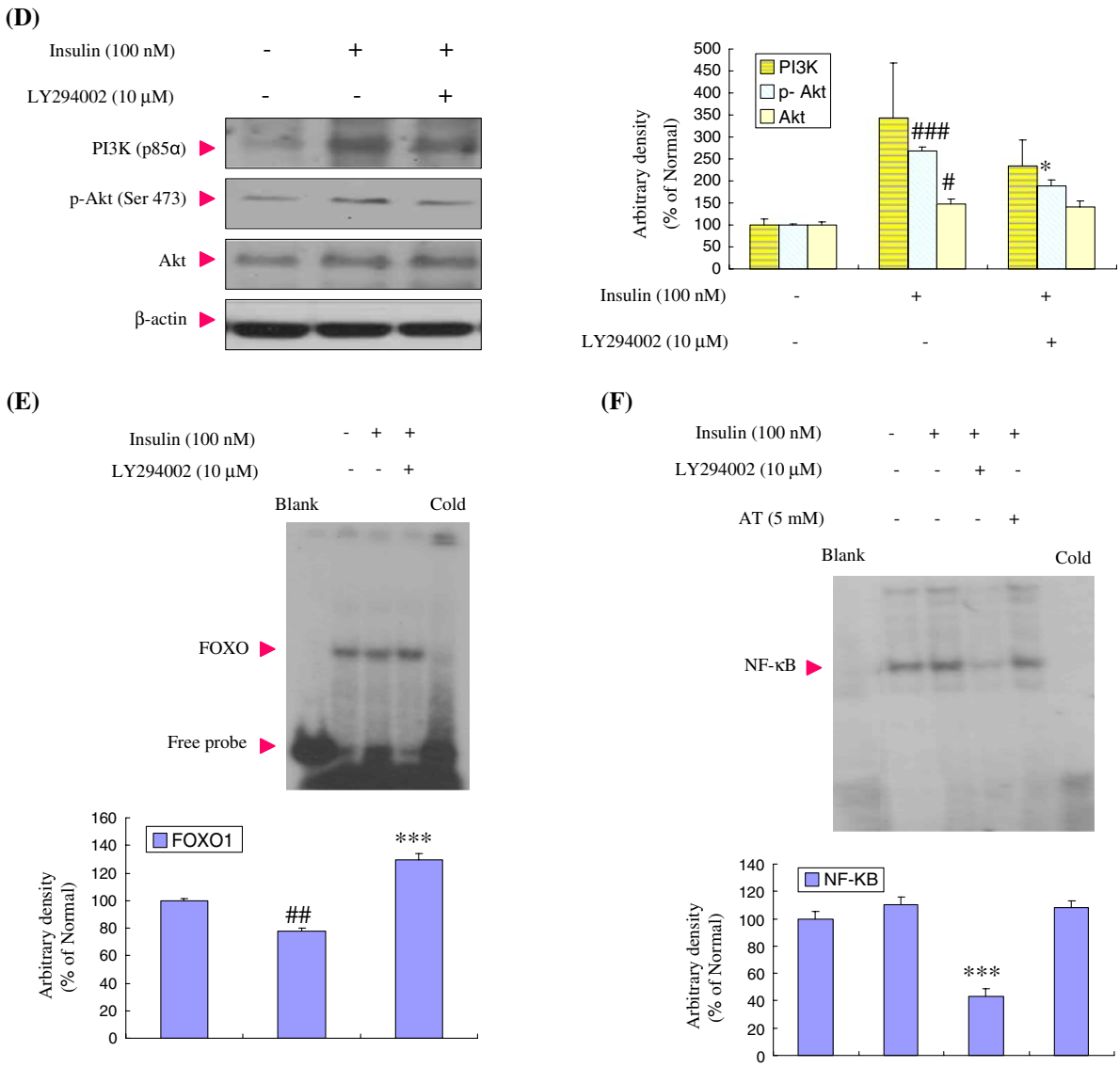

Fig. 4 continued

The ability of insulin to modulate transcription in wild type or mutant-FOXO1 was further examined using HEK293T cells. Cells were transfected with wild- or mutant-FOXO1-expressing plasmids and then were monitored for NF-κB-dependent luciferase expression to delineate the action of NF-κB in FOXO1 activation. Results shown in Fig. 6b indicated that in the absence of insulin, wild- or mutant-FOXO1 stimulated NF-κB activity is approximately 2.2- and 1.1-fold, respectfully, compared to the NF-κB-transfected control. Insulin inhibited transcription activity stimulated by the mutant-FOXO1 transfection, while increased transcription activity was stimulated by wild-type FOXO1 transfection (Fig. 6b).

To determine the extent of FOXO1-related modulation of NF-κB, EMSA was carried out with nuclear proteins. Results showed insulin-stimulated wild-type FOXO increases in the nuclear binding activity of NF-κB in HEK293T cells. On the other hand, insulin-stimulated mutant-FOXO1 did not change NF-κB binding activity (Fig. 6c). The binding specificity of the FOXO complex was demonstrated

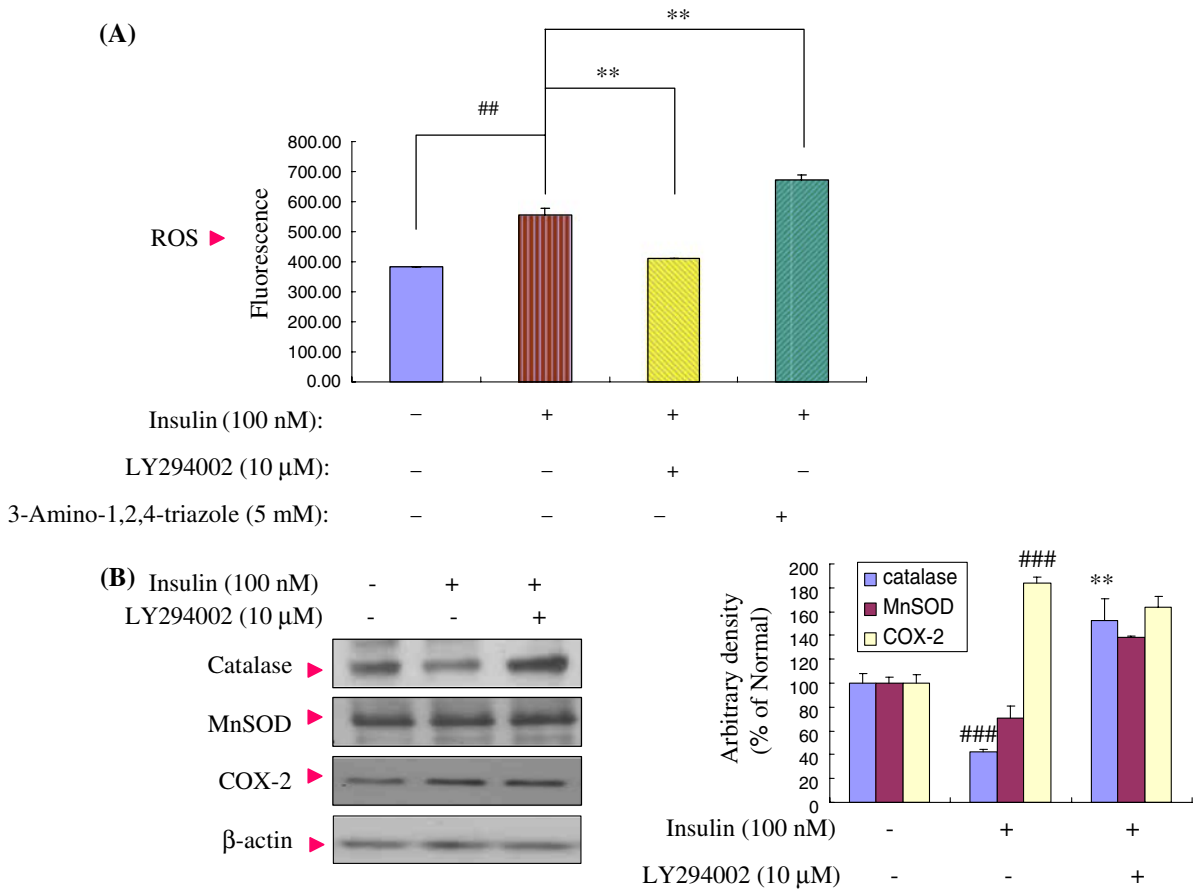

Fig. 5 Insulin induced oxidative stress in HEK293T cells. (**a**) Quantitative analysis of fluorescence intensity using DCFDA was detected after treatment with vehicle or 10 μM insulin in the absence or in the presence of PI3K/Akt inhibitor (LY294002, 10 μM) and catalase inhibitor (3-amino-1,2-4-triazole, 5 mM) for 1 h. As a statistical significance, results of one-factor ANOVA: $^{\#\#}P < 0.01$ vs. insulin untreated group; $^{**}P < 0.01$ vs. insulin treated group, respectively. (**b**) After stimulation with insulin in the absence (−) or presence (+) of LY294002 (10 μM) and catalase inhibitor (3-amino-1,2-4-triazole, 5 mM), the cells lysed and were extracted from cytoplasmic. The Western blot was performed for COX-2, catalase, and MnSOD. Samples loaded on the sample gel were probed with β-actin. Results of one factor ANOVA: $^{\#\#\#}P < 0.001$ vs. insulin untreated group; $^{**}P < 0.01$ vs. insulin treated group, respectively

using a 100-fold excess of an unlabeled oligonucleotide (Fig. 6c, Lane 8). Data indicated that constitutive activation of Akt by insulin increased the induction of NF-κB transcription activity by a phosphorylated FOXO1-dependent mechanism and thereby promoted age-related inflammatory gene expression.

Discussion

Initial clues that PI3K controls FOXO activity came from studies performed with the nematode, *C. elegans*. Genetic studies established that PI3K suppresses the function of DAF-16, a Forkhead transcription factor (Lin et al. 1997). This was found to be critical for proper control of metabolism and cell survival (Birkenkamp and Coffer 2003). At molecular levels, FOXO proteins bind to IRE in the proximal promoter (Durham et al. 1999; Tang et al. 1999) of target genes involved in cell survival, cell cycles, DNA repair, insulin sensitivity (Brunet et al. 1999; Birkenkamp and Coffer 2003) and stimulate their transcription. Recently, NF-κB was found to mediate the induction of the MMP-9 production by Akt (Kim et al. 2001), which is activated in response to oxidant injury as well as several other stresses known to exert their cytotoxic

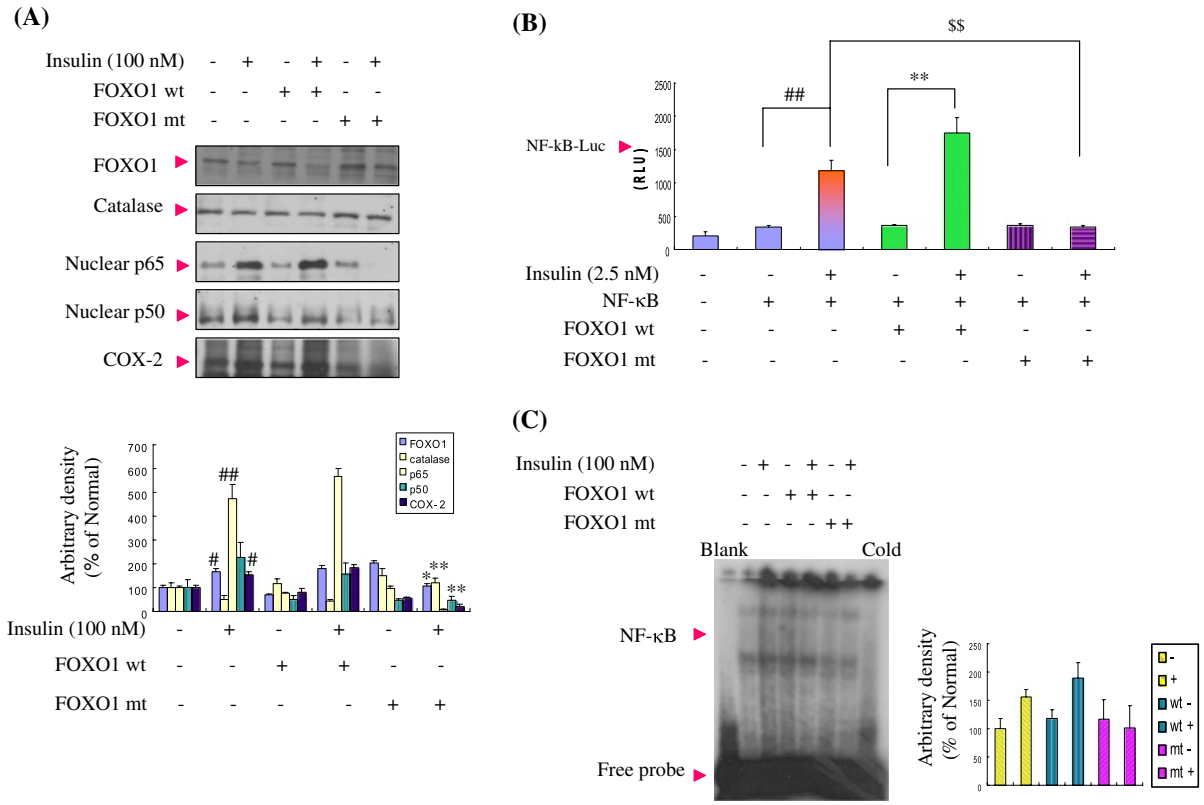

Fig. 6 Effect of FOXO1 on insulin-induced NF-κB activation in HEK293T cells. (**a**) Cells were cultured in presence or absence of insulin. Cells were transduced with wild or mutant-FOXO1 plasmids overnight. Levels of catalase, p65, p50, and COX-2 protein were determined with specific antibodies. Results of one factor ANOVA: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. insulin untreated group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. insulin treated group, respectively. (**b**) Lysates of 293T contranfected with NF-κB-Luc and wild or mutant-FOXO1 indicate insulin treatments were subjected to luciferase assay. As a statistical significance, results of one-factor ANOVA: $^{\#\#}P < 0.01$ vs. insulin untreated NF-κB-Luc transfection control; $^{**}P < 0.01$ vs. insulin untreated NF-κB-Luc and wt-FOXO cotransfection group; $^{\$\$}P < 0.01$ vs. insulin treated NF-κB-Luc transfection group, respectively. (**c**) HEK293T cells were transiently transfected with FOXO1 wild-type or mutant. Nuclear extracts were assayed in band shift experiments using the FOXO as the ^{32}P-labeled oligonucleotide

effects in part through the generation of ROS or perturbations in cellular redox status (Huang et al. 2001).

NF-κB, a ubiquitous proinflammatory transcription factor known to be activated by a wide variety of stimuli including ROS and oxidative stress (Pahl 1999), was identified as a functional target of Akt (Ozes et al. 1999). Studies show that the activation of redox-sensitive NF-κB plays a pivotal role in modulating the cellular signaling mechanism for oxidative stress-induced inflammation during aging. Decreases in inhibitory IκBα protein levels showed the activation and increased nuclear translocation of NF-κB during aging in AL rats, and which were attenuated by CR (Fig. 2d).

In the present study, we documented a new finding that the upregulation of FOXO1 phosphorylation and NF-κB activities that occur during aging can be prevented by the anti-oxidative CR regimen. This new finding may provide a link between insulin signaling and inflammation. This study also showed that serum insulin levels increases with aging and that CR suppresses these increases. Changes in the insulin level and its modulation deem to be significant because the decreased FOXO binding activity that results during aging is blunted by activated PI3K/Akt, which is insulin-dependent. As expected and shown in Fig. 1, the insulin-suppressive action of CR prevented the age-dependent decrease of FOXO1 by inhibiting the PI3K/Akt pathways.

Ad libitum feeding and aging are well-known causes of increased 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, leading to translocation of these proteins from the nucleus into the cytoplasm (Brunet et al. 1999). It was reported the inhibition of the FOXO and other related proteins through phosphorylation by Akt (Tang et al. 1999). Moreover, Lin et al.(2004) reported that FOXO can inhibit NF-κB activation, suggesting 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. Although a large number of PKB substrates have been implicated in the regulation of cellular survival (Lawlor and Alessi 2001).

Emerging evidence has shown how PI3K/Akt signaling may regulate cellular levels of ROS (Matsumoto et al. 2006; Erol 2007). Recently, investigators have also shown that Akt-regulated FOXO can reduce the level of cellular oxidative stress by directly increasing mRNA and protein levels of MnSOD and catalase (Burgering and Medema 2003). Akt-mediated phosphorylation of FOXO1 results in the translocation of FOXO1 from the nucleus to the cytosol. Consequently, Akt activation decreases catalase levels, and this is likely to contribute to an increase in cellular ROS.

To substantiate in vivo data, we examined the modulation of FOXO1 by insulin signaling in cultured HEK293T cells. We found that when cells were exposed to insulin, the PI3K/Akt pathway is activated, resulting in the phosphorylation of FOXO1 (Thr 24, Ser 256) and in the down-regulation of the target gene expression (Fig. 5b). These data suggest that insulin stimulated Akt-mediated phosphorylation of FOXO1. Consistent with these findings, we found that the phosphorylation of Akt and FOXO1 is reduced in a parallel fashion by a known inhibitor of PI3K/Akt, LY294002 (Fig. 4a, d).

While examining how insulin affects FOXO expression upon NF-κB activation, we found that proinflammatory NF-κB expression increased to a greater extent in insulin-stimulated wild-type FOXO1, compared with mutant FOXO1. Moreover, it was found that insulin increased NF-κB activity via a FOXO/catalase mechanism in HEK293T cells. These results strongly indicate that insulin is capable of activating NF-κB when the nuclear export of the FOXO1 is phosphorylated. We also found that PI3K/

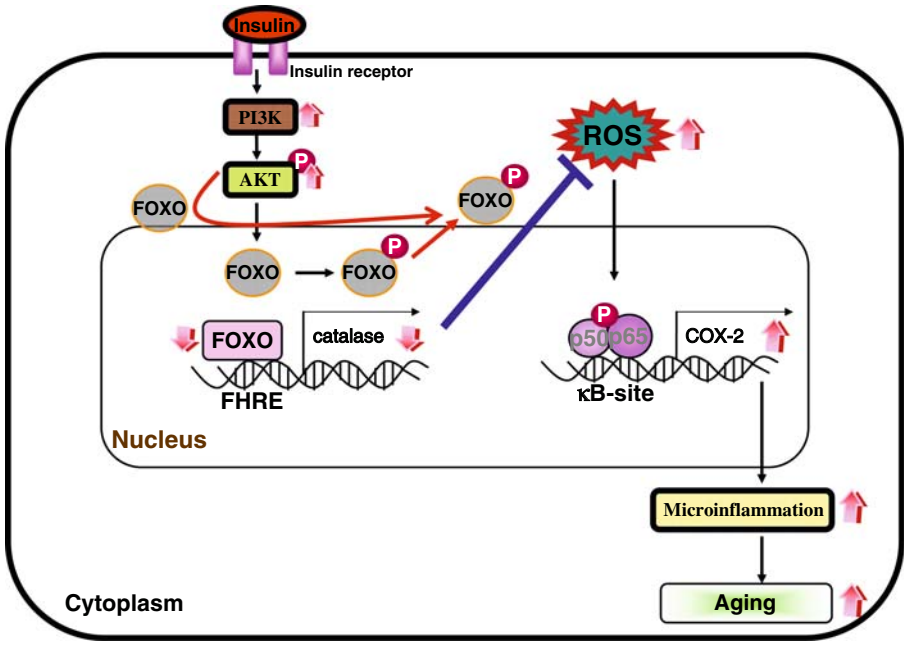

Fig. 7 Possible mechanisms of aging and CR in Akt/FOXO/NF-κB pathway. PI3K, phosphatidylinositiol 3-kinase; FOXO, Forkhead transcription factor; ROS, reactive oxygen species; NF-κB, nuclear factor-kappa B; COX-2, cyclooxygenase-2

Akt positively regulates NF-κB in HEK293T cells and during aging via the FOXO1 pathway and that this action might be mediated at least in part by the FOXO1 that regulates cellular ROS levels via catalase. Furthermore, we were able to show that NF-κB is a direct target of PI3K/Akt-induced FOXO1 activation and that phosphorylated FOXO1 inhibits target gene catalase during aging. More significantly, FOXO1 mutation suppressed NF-κB activation (Fig. 7).

Recent reports suggest that in hepatocytes, the inhibition of PI3K activity by both specific inhibitors (Wortmannin and LY294002) abrogated FOXO1/3 phosphorylation by insulin (Mounier et al. 2006). Moreover, insulin modulates many aspects of the inflammatory network (Dandona et al. 2002). Azevedo-Martins et al.(2003) reported that the activation of NF-κB is suppressed through an overexpression of the mitochondrial isoform of SOD when using RINm5F insulin-producing model cells. These data support our data that insulin stimulated the proinflammatory reaction by FOXO1 phosphorylation.

The significance of the current findings is multifaceted: the age-related decrease in FOXO1 transcriptional activity is due to increased phosphorylation; and PI3K/Akt and NF-κB activation increase during aging, all which may closely be related to the increased levels of insulin with age. More importantly within the context of aging, theses age-related changes were counteracted by the anti-aging action of CR. In this regard, it is interesting to note a recent paper that hypothesized that insulin resistance is an evolutionarily conserved protection against age-related oxidative stress (Erol 2007). Based on our data observed from current in vivo and in vitro studies, we propose that the age-related phosphorylation of FOXO1 induces NF-κB activation through repression of catalase expression. It is further proposed that both these transcription factors are regulated by PI3K/Akt activation through age-related insulin enhancement, which in-turn can be modulated by the anti-aging action of CR.

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