Manganese-superoxide dismutase (MnSOD) is one of the major cellular antioxidant defense systems. To study the effect of age on the regulation of MnSOD in the vasculature, we compared MnSOD expression and its transcriptional regulation in explanted vascular smooth muscle cells (VSMC) isolated from old (24 months old) versus young (6 months old) rats and grown in a normal (5 mM) or high (12.5 and 25 mM) glucose or tumor necrosis factor α (5 ng/ml) environment to induce oxidative stress. Both MnSOD protein and activity were reduced in VSMC from old compared with young animals. FOXO3a, a member of the family of Forkhead transcription factors, interacted with the promoter of the rat MnSOD gene at a specific binding site. Inhibition of FOXO3a transcription with small interfering RNA led to a reduction in MnSOD gene at a specific binding site. Inhibition of FOXO3a transcription with small interfering RNA led to a reduction in MnSOD gene expression. VSMC from old rats had increased phosphorylated FOXO3a at Ser253, which paralleled the reduction of MnSOD protein. Treatment of VSMC with 5 mM insulin-like growth factor-1 induced phosphorylation of Akt and FOXO3a over time, repressing FOXO3a DNA binding and consequently MnSOD gene expression. Furthermore, Akt activity was selectively increased in VSMC from the old, supporting the hypothesis that increased age-related Akt activity might be responsible for the phosphorylation and inactivation of FOXO3a, which in turn down-regulates MnSOD transcription.

Scientists have suspected for half a century that reactive oxygen species play a major role in the aging process and the pathogenesis of age-related disease. Numerous studies underscore the importance of dysregulated oxidant and antioxidant balance in advancing age (1) and in the development and progression of atherosclerosis both in animal models and in humans (2). MnSOD, also known as superoxide dismutase-2 (SOD2), represents a major cellular defense against superoxide free radicals in the mitochondria (3). The regulation of MnSOD during the aging process is still unclear. Some studies report lower levels of MnSOD expression and activity in aged rat brain and liver (4) as well as adrenal and testicular cells (5, 6), whereas other investigators have reported increased MnSOD activity with aging, especially in rat myocardium (7) and human skeletal muscle (8), implicating tissue and cell type specificity. Nevertheless, MnSOD activity in VSMC from aged mice (1) and in human arteries from older individuals (9) was reported to be significantly reduced.

The transcriptional regulation of the MnSOD gene is complex. A series of transcription factors, such as specificity protein-1, activator protein-2, and NF-κB, appear to regulate MnSOD gene expression (10–12). Recently, a Forkhead transcription factor, DAF-16, was reported to be involved in the regulation of longevity in C. elegans (13). In the search for candidate genes for DAF-16, sod3 (MnSOD homologue in C. elegans) was found to be directly regulated by DAF-16 (14). FOXO (Forkhead box class O) transcription factors may be important in their ability to regulate antioxidant defense in many species. For example, FOXO3a, also known as FKHR1, protects quiescent human cells from oxidative stress by directly increasing the quantities of MnSOD mRNA and protein (15).

FOXO transcription factors are believed to be negatively regulated by IGF-1 signaling pathway through phosphatidylinositol 3-kinase and protein kinase B, also called Akt (16, 17). In addition, FOXO transcriptional activity may be enhanced by β-catenin, which binds directly to FOXO in cells exposed to oxidative stress (18).

A model depicting the FOXO shuttling system was initially described to explain the mechanism of transcriptional regulation by this family member of transcription factors (19). This shuttling system changes the intracellular localization of FOXO through phosphorylation of three Akt sites in the Forkhead domain, primarily at Ser253. In an in vitro Akt kinase assay, Brunet et al. (16) tested a series of GST-FOXO3a fusion proteins in which each of the three phosphorylation sites, Thr32,
Ser253, and Ser315, were mutated to Ala and confirmed that the Ser253 mutant GST-FOXO3a fusion protein was unable to be phosphorylated by Akt. Phosphorylation of FOXO3a at Ser253 in the Forkhead domain leads to disruption of the nuclear localization function and its binding to DNA (20). After phosphorylation of FOXO3a, the complex that includes 14-3-3 proteins, a small GTPase Ran, and chromosomal region maintenance protein 1, is transported through the nuclear pore into the cytosol (21). In the cytosol, phosphorylated FOXO proteins are degraded by the ubiquitin-proteasome system. This shuttling system provides the cell with the “double negative” regulation of FOXO factors and the subsequent regulation of transcription (22). Understanding the role of Akt and FOXO3a in the regulation of MnSOD in VSMC with advancing age or under conditions known to promote atherosclerosis would be important as future targets for attenuating oxidative stress and its consequences. We hypothesized that MnSOD expression in VSMC from young and old rats might differ as a consequence of differences in age-related regulation of FOXO3a phosphorylation by Akt during oxidative stress that was simulated by exposure to high glucose (23, 24) or the addition of TNF-α (25, 26).

EXPERIMENTAL PROCEDURES

Explanted Aortic VSMC—Explanted VSMC isolated from young and old rats were cultured as described previously (27). Young mature (6 month old) and old (24 month old) male Fisher 344 rats were purchased from Harlan-Sprague-Dawley, Inc. (Indianapolis, IN). The rats were originally obtained from the NIA, National Institutes of Health aging colonies and maintained in separate security barriers at Harlan Sprague-Dawley according to the guidelines established in the Guide for the Care and Use of Laboratory Animals and under specific pathogen-free conditions. After exsanguination, the aortas were surgically removed and cleaned of superficial adherent connective tissues. Then the aortas were minced into small pieces and placed in 6-well plates with Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen), supplemented with 10% fetal bovine serum and 1 unit/ml penicillin/streptomycin. VSMC migrated from the tissue after 3–4 days at 37 °C incubation in an atmosphere of 10% CO2. At confluence, monolayers of cells were dispersed with 0.25% trypsin and then seeded at 0.3–0.5 × 10^6/100-cm² flask.

VSMC at passages 3 or 4 were used in these experiments. Pairs of cell lines from one old and one young rat were grown and treated under identical conditions in each experiment as previously described (27). VSMC were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin containing normal glucose (5 mM) or high glu-
cose (12.5 or 25 mM) for 3 days. Another group was exposed to TNF-α/H9251 (5 ng/ml) in reduced DMEM (DMEM supplemented with 2% fetal bovine serum, 0.2% bovine serum albumin, and 1% penicillin/streptomycin) for 3 h to induce oxidative stress.

**IGF-1 Treatment**—IGF-1 protein (Abcam Inc, Cambridge, MA) was diluted to a 1 μM stock solution in phosphate-buffered saline buffer. VSMC from young rats were grown in complete DMEM overnight after subculturing. On the second day, the medium was changed to reduced medium with 5 nM IGF-1 for 1, 2, 4, 6, 8, or 24 h. A parallel set of VSMC in reduced medium without IGF-1 were used as controls.

**AG-1024 Treatment**—VSMC were grown in complete DMEM overnight after subculturing. The next morning VSMC were pretreated for 30 min with 10 μM AG-1024 (Calbiochem, La Jolla, CA), an IGF-1R inhibitor. Then the medium was changed to reduced medium containing 5 mM IGF-1 with or without 10 μM AG1024 for 4 h. Alternatively, VSMC from old rats were grown in complete DMEM containing 5 mM glucose overnight. On the second day, one set of dishes was pretreated with 10 μM AG-1024 for 30 min and then continuously grown in the medium containing 5 mM or 12.5 mM glucose with 10 μM AG-1024 for 3 days. The other set of cells were grown in the same medium without AG-1024 but with 1 μl/ml Me2SO as the vehicle control.

**siRNA Treatments**—FOXO3a siRNA SMARTpool was synthesized by Dharmacon, Lafayette, CO according to rat LOC294515 (XM_215421) FOXO3a (predicted). VSMC were seeded into 100-mm dishes, 6-well plates, or Nunc Lab-Tek II™ chamber slides (Nalge Nunc International, Rochester, NY) at 0.7 × 10⁶/dish, 1 × 10⁵/plate, and 5 × 10⁴/well, respectively. After 24 h, the siRNA was diluted to a 2 μM working solution and delivered to cells at 100 nM final concentration through a lipid-mediated DharmaFECT Transfection Reagent (Dharmacon). A siGLO Cyclophiling B siRNA (Dharmacon) was also included as a transfection control.

**Western Blots**—Antibody for MnSOD was from OXIS (Foster City, CA); antibodies for FOXO3a, phospho-FOXO3a (Ser253), Akt, phospho-Akt (Thr308), and (Ser473) were from Cell Signaling (Danvers, MA); the monoclonal antibody for the IGF-1R chain was from Biomol (Plymouth, PA) and for -actin from Abcam. Cytoplasmic and nuclear proteins were extracted from VSMC as described previously (27). 15 μg of each nuclear protein or 20 μg of each cytoplasmic protein was electrophoresed on 10% SDS-PAGE gels and then electroblotted onto nitrocellulose membranes. The membranes were incubated with the appropriate primary antibody overnight with shaking at 4 °C. After incubation with corresponding secondary antibodies, the protein bands were visualized using a SuperSignal™ West Pico Trial Kit (Pierce) and exposed to radiographic film. The blots

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**FIGURE 2.** A, EMSA results show DNA binding activity in the search for FOXO3a-binding site in the rat SOD2 promoter. Double-stranded oligonucleotides: rSOD2-P1, rSOD2-P2, DBE consensus, and rSOD2-P2Mut were labeled with [γ-32P]ATP and used for binding assays with nuclear extracts from VSMC. Lanes 1 and 2, rSOD2-P1; lanes 3 and 4 and 7–11, rSOD2-P2; lanes 5 and 6, rSOD2-P2Mut. In addition, lane 8 shows a supershift with an anti-FOXO3a antibody, lane 10 shows competition with the rSOD2-P2Mut, and lane 11 shows competition with the DBE consensus. B, on the left side, an agarose gel image shows the PCR products amplified from purified ChIP DNA. Lane 1, a negative control by anti-mouse IgG; lanes 2–4, FOXO3a-bound chromatin; lane 5, and a ChIP quality control by anti-RNA polymerase II. In addition to the ChIP DNA, lane 5 is a ChIP input; lanes 7 and 8 show PCR bands from rat genomic DNA as a band size control, and lane 9 shows shear efficiency. The table on the right displays the samples, antibodies, primer combinations used, and predicted PCR sizes corresponding to each lane. The bottom scheme shows the primer positions to the DBE binding site in the rat SOD2 promoter.
FOXO3a Regulation of MnSOD with Advancing Age

A FOXO3a Binding to SOD2 (EMSA)

|          | Young | Old |
|----------|-------|-----|
| Glucose (mM) |       |     |
| 5        | 125   | 25  |
| 12.5     |       |     |
| 25       |       |     |

B Relative Value (%) for FOXO3a Binding to SOD2

FIGURE 3. A, VSMC from 3 pairs of young (6 months old) and old (24 months old) rats were grown in DMEM containing 5 (normal control), 12.5, or 25 mM glucose for 3 days or exposed to 5 ng/ml TNF-α for 3 h prior to extraction of nuclear proteins. On the left, a representative EMSA result shows FOXO3a binding activity using the rSOD2-P2 probe. On the right side is shown an EMSA gel run with rSOD2-P2 and OCT-1 probes (loading control) together. B, a bar graph summarizes the FOXO3a binding activities in VSMC from young (lightly shaded bars) and old (darkly shaded bars) rats, normalized to OCT-1. The p value is for comparison between young and old (*, p = 0.05).

were reprobed with a β-actin antibody to detect nuclear (28) or cytoplasmic β-actin as the loading controls. The images and densities were captured with a GS-700 imaging densitometer (Bio-Rad) and analyzed with Quantity One software version 4.2 (Bio-Rad).

MnSOD Activity Assay—MnSOD activity was visualized by a method modified from the classical nitro blue tetrazolium staining (29). Briefly, 60-μg protein samples were loaded onto the 12% native gel in 1 × Tris-glycine running buffer and run at 120 V. After electrophoresis, the gels were stained with 2.4 mM nitro blue tetrazolium (Sigma-Aldrich) containing 28 mM flavin and 28 mM TEMED in the dark for 30 min. The gels were illuminated under bright fluorescent light for 30 min and sandwiched in transparent paper for image scanning. Coomassie Brilliant Blue stain was used to determine protein loading.

Electrophoretic Mobility Shift Assay (EMSA)—Double-stranded oligonucleotides from the rat MnSOD gene (rat SOD2, X56600) promoter region, DBE consensus (DBECons), rSOD2 mutant, and octamer-1 (OCT-1, J00565) with a OCT-1 consensus binding site were synthesized by Invitrogen: rSOD2-P1a, 5'-CTCAATAACTTGGATTATTATGAGA-3'; rSOD2-P1b, 5'-CTCTGTATTAAACGAGGTATTTGAG-3'; rSOD2-P2a, 5'-ACATGTTGCTAACCACAAATTAAGAGGAA-3'; rSOD2-P2b, 5'-TTCTTTTAATTTGTTAGTTGAGCA-TACTGT-3'; DBECons, 5'-AATTATACCTTGTGTGTACTTTAC-3'; DBEConsb, 5'-GATGTAATTAAAAGAGGA-3'.

were incubated with 1:400 diluted Cy3-Goat anti-rabbit IgG (Jackson, West Grove, PA) or Alexa Fluor 488 anti-mouse IgG (Molecular Probes, Eugene, OR) at room temperature for 2 h, followed by 15 min with DAPI (Sigma-Aldrich). The slides were finally examined using confocal laser scanning microscope LSM 510 (CSLM, from Carl Zeiss Inc., Thornwood, NY) and scanned for Cy3 staining (560–615 nm), AF 488 (505–530 nm), and DAPI (420–480 nm).

Akt Kinase Assay—An Akt Kinase Assay Kit was obtained from Cell Signaling in which an immobilized Akt monoclonal antibody was designed preferentially for phosphorylated Akt at serine 473 (P-Akt). Briefly, Akt was isolated from 50 μg of nuclear proteins from each treatment through immunoprecipitation (IP) overnight with 20 μl of immobilized Akt antibody at 4 °C. Following multiple washings with cell lysis buffer and kinase buffer supplied by the kit, the pellet with bound phosphorylated Akt was suspended in 50 μl of glycogen synthase kinase-3 (GSK-3) fusion protein and then incubated for 30 min at 30 °C. After centrifugation at 14,000 × g for 30 s at 4 °C, the samples were heated and loaded onto SDS-PAGE gels for further processing of Western blots. The phosphorylated GSK bands were then detected by phospho-GSK-3a/b (Ser21/9) antibody and compared with total GSK.

Chromatin Immunoprecipitation (ChIP) Assay—VSMC histones were cross-linked to DNA by adding 1% formaldehyde
directly to the culture medium for 15 min at 37 °C. The ChIP assay kit was from Upstate (Chicago, IL). The cells were lysed with 200 μl of SDS lysis buffer/106 cells, containing protease inhibitors. Cell lysates were sonicated to shear DNA in a range of 200–1000 bp with a 600-watt ultrasonic processor setting at 30% of maximum power. Cross-links in part of the chromatin were recovered for visualization of shearing efficiency on an agarose gel. Twenty μl of chromatin in the supernatant of sonicated cell lysates were saved as input/starting material, whereas the rest of the chromatin in the supernatant was diluted in a 2-ml volume with ChIP dilution buffer, which was then precleaned by protein agarose slurry. FOXO3a-bound chromatin was then immunoprecipitated by 4 μg of antibody for FOXO3a from Santa Cruz (Santa Cruz, CA) and then dissociated with proteins through the procedures recommended by the manufacturer. In addition, anti-mouse IgG and anti-RNA polymerase II antibodies were also included in the IP to process DNA for a negative control or a ChIP quality control, respectively. The purified DNA (ChIP DNA) from FOXO3a-bound chromatin and controls was finally used as a template for PCR amplification. In addition to rsOD2-P1a and rsOD2-P2b, two other primers used in the rat SOD2 promoter (X56600) were: SOD2P-F, 5′-TCA-TGGCCACTATGCTCAA-3′; and SOD2P-R, 5′-TCTCAAGCTGTGTCCTCTTCT-3′. The primers spanning glyceraldehyde-3-phosphate dehydrogenase (NM_017008), which was cross-linked to acetyl histone H3, were also designed for quality control DNA: GAPDH-F, 5′-CATTTACCTCAACTACATGG-3′; and GAPDH-R, 5′-TGACCAGCTTCCTATTCTCA-3′. Up to 1 μg of template was added to 50 μl of PCR mixture containing 1.25 units of AmpliTaq Gold (Applied Biosystem, Foster City, CA), 0.2 mM dNTPs, 1× PCR buffer with MgCl2, and 200 nM of each primer. PCR was run under the following conditions: 94 °C for 14 min for 1 cycle; 94 °C for 1 min, 68 °C for 1 min, 72 °C for 2 min for 14 cycles; 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min for 20 cycles; and a final extension at 72 °C for 8 min.

RT-PCR—Total RNA was isolated from control and siRNA-treated VSMC with UltraspecTM-II (Bio- tecx, Houston, TX). RT-PCR primer sets for FOXO3a (XM_215421) and MnSOD (NM_017051) mRNA were specially designed crossing exons: FOXO3a-F, 5′-GCAAAACGGCTTCGTT-3′; FOXO3a-R, 5′-TCGGCTCTTGTTGACTTG-3′; MnSOD-2F, 5′-CACTCAAGCTGTGTCCTCTTA-3′; and MnSOD-R, 5′-CGACCTGTGTCCTATTGA-3′. QuantumRNA™ Universal 18 S primers from Ambion (Austin, TX) was mixed with the supplied Competimer at the ratio of 3:7 and used as internal control. One μg of total RNA was added in 20 μl of reverse transcription mixture containing 1 μl of MMLV reverse transcriptase (Invitrogen) and 1× MMLV RT buffer, 1 μl of random primers (Invitrogen), 0.25 mM dNTPs, 10 mM dithiothreitol and incubated at 42 °C for 70 min followed by at 98 °C for 3 min. After reverse transcription,
1–2 μl of cDNA was loaded into a total PCR mixture (20 μl) composed of 0.5 unit of Ampli Taq Gold, 1× PCR buffer with MgCl₂, 0.2 mM dNTPs, 22.5 nM of each forward or reverse primers, and 1 μl of 18 S primer mixture. PCR was run under the same condition as described above and then visualized on 2% agarose gels.

RESULTS

Age Reduces MnSOD Protein and Enzyme Activity in VSMC—As shown in Fig. 1 (A and B), MnSOD protein levels in VSMC from old rats were ~35% lower than that found in young rats when VSMC were grown in control medium (5 mM glucose) for 72 h and ~44% lower than young when grown in 12.5 mM glucose medium. At supraphysiologic levels of glucose (25 mM) or after the addition of TNF-α, there were no overall effects of age on MnSOD. Post hoc analysis revealed significant differences between young and old under control and high physiologic glucose conditions (p < 0.02). Using the nitro blue tetrazolium native gel assay to determine the catalytic activity of MnSOD, VSMC from old rats consistently showed lower activity under all conditions when compared young rats (Fig. 1C). These data suggest that VSMC from old rats have an overall lower protein content and activity of MnSOD under basal (5 mM) glucose or moderately higher (12.5 mM) glucose. In contrast, MnSOD levels and activity in cells from the young rats did not appear to change in response to high glucose or TNF-α, suggesting sufficient capacity to handle oxidative stress induced by these agents.

Evidence for FOXO3a Binding to SOD2 Promoter—Because our goal was to determine the relationship between the regulation of FOXO3a and the transcription of MnSOD, we next demonstrated that direct binding of FOXO3a to the MnSOD gene occurs in the rat, as has been reported in human cells (15). 9.8 kb of the rat MnSOD gene was previously isolated and sequenced (31). A core sequence (TTGTTTAC), also known as a consensus of DAF-16 family protein-binding element (DBE) (14), then was used to search 2 kb of the rat SOD2 promoter, including another 5-kb upstream segment of the SOD2 in rat chromosome 1 genomic contig (NW_047553). No identical sequence was found, but two suboptimal sequences, each with one nucleotide difference, were obtained. One was TTGTTTAT located at 990–997 in SOD2, and the other was an inverse of TTGTTTAG located at 1309-1302.

Based on the adjacent sequence of the SOD2 promoter, we were then able to design oligonucleotides for a DNA binding assay. Two 32-mer double-stranded oligonucleotides were synthesized, rSOD2-P1 and rSOD2-P2, each containing one of the binding sites. The pool of phosphorylated FOXO3a to FOXO3a ratio is expressed as a percentage of young control (mean ± S.E.). p value is for comparison between young and old. *, p < 0.05.

FIGURE 5. VSMC from four pairs of young (6 months old) and old (24 months old) rats were grown in DMEM containing 5 (normal control), 12.5, or 25 mM glucose for 3 days or exposed to 5 ng/ml TNF-α for 3 h. Western blot analysis for FOXO3a in cytoplasmic and nuclear protein extracts from VSMC is shown. A, two representative Western blots of nuclear and cytoplasmic proteins. Each blot was sequentially probed with antibodies for FOXO3a (top panels), phospho-FOXO3a at Ser253 (middle panels), and β-actin (lower panels). B, summary of phosphorylated FOXO3a in the nucleus for young (lightly shaded bars) and old (darkly shaded bars) rats. The pool of phosphorylated FOXO3a to FOXO3a ratio is expressed as a percentage of young control (mean ± S.E.). p value is for comparison between young and old. *, p < 0.05.
or rSOD2 mutant with a TTATTTAC site (lanes 5 and 6). The binding of nuclear protein to rSOD2-P2 could be competed by the DBE consensus sequence (lane 11), but not by the mutant version (lane 10). These data demonstrate that nuclear protein from VSMC can bind to the SOD2 promoter DNA in a cell-free binding assay. A supershift assay using an antibody for FOXO3a showed a relatively light band (lane 8), but no shifted band was observed. To further confirm that binding occurs between the FOXO3a protein and the SOD2 promoter, two primer sets adjacent to TTGTTTAG were designed for use in a more powerful ChIP assay. Primer F and R were 217 bp apart, whereas primer 1a and 2b were 345 bp apart (Fig. 2B). The native binding complex was preserved through chromatin cross-linking before collecting the cells, and FOXO3a-bound chromatin was obtained through IP. The results show that the exact sizes of PCR products, as predicted using the primer combinations, were amplified from the purified CHIP DNA (Fig. 2B). These studies for the first time identified a specific DBE site for FOXO3a binding to the rat SOD2 promoter.

Effect of Age on FOXO3a Binding to the SOD2 DNA—Having demonstrated FOXO3a binding to SOD2 at the particular DBE site, we were then able to compare the binding activity between young and old VSMC using a DNA binding assay. An EMSA using only the rSOD2-P2 probe (Fig. 3A, left panel) showed that FOXO3a binding activity was lower in nuclear extracts from old VSMC grown in control and 12.5 mM glucose media but equal to or slightly higher when grown in 25 mM glucose or TNF-α. Because the EMSA approach does not easily lend itself to quantification for the lack of a loading control, a DNA probe containing an OCT-1-binding site was used. When the binding assay was run with the two probes, two distinguishable binding bands were seen (Fig. 3A, right panel). Normalizing to OCT-1 binding, the differences in the binding activity between young and old were confirmed but only significant under control (5 mM glucose) conditions (p = 0.04), as shown in Fig. 3B.

FOXO3a Controls MnSOD Transcription—Thus far we have shown that old age is associated with lower MnSOD protein and activity as well as lower FOXO3a binding to SOD2 DNA in explanted VSMC. To establish whether FOXO3a is causally involved in transcriptional regulation of the MnSOD gene, the siRNA technique was used and inhibited FOXO3a gene expression as shown in the representative CSLM image in Fig. 4A. FOXO3a protein in VSMC transfected with a scrambled construct was abundant in VSMC the perinuclear region and within the nucleus. Reduction was evident when VSMC were treated with FOXO3a siRNA. The upper panels of Fig. 4B further demonstrated that the reduction of FOXO3a RNA was ~70% using semi-quantitative RT-PCR, and the reduction of nuclear FOXO3a protein was ~58% by Western blot analyses. As expected, MnSOD RNA and protein levels were also lower with FOXO3a siRNA inhibition, ~49% reduction in RNA and ~51% reduction in protein, respectively, as shown in the lower panels of Fig. 4B. Furthermore, the corre-
lated changes were confirmed by the EMSA result shown in the far right panel of Fig. 4B that shows only ~46% of FOXO3a binding activity remained using the siRNA approach. In summary, these data not only provide proof that FOXO3a controls MnSOD transcription but also demonstrate consistent changes between MnSOD mRNA expression and protein levels using the same approach.

Effect of Age on Phosphorylation of FOXO3a—Having demonstrated that FOXO3a plays a role in the regulation of MnSOD gene expression, we next used Western blot analysis in both nuclear and cytosolic extracts from old versus young VSMC to demonstrate differences in the localization of FOXO3a. VSMC from the young rats had higher levels of FOXO3a in the nucleus compared with the cytosol under control (5 mM glucose) conditions (Fig. 5A, top panel). When these blots were reprobed with an antibody for phosphorylated FOXO3a at Ser253 (P-FOXO3a), a marked increase in P-FOXO3a was found in both nuclear and cytosolic extracts of VSMC from old versus young rats (Fig. 5A, middle panel). The changing nuclear pool of P-FOXO3a to FOXO3a ratio for both age groups (Fig. 5B) confirms that there was an ~3.5-fold increase in P-FOXO3a in VSMC from old rats versus young under control conditions and ~1.8-fold change in P-FOXO3a under the 12.5 mM glucose condition (p < 0.05). These data are in agreement with the proposed model, i.e. higher P-FOXO3a in VSMC from old rats causes reduced FOXO3a transcriptional activity, thereby paralleling the lower MnSOD protein levels and activity seen in Fig. 1.

Upstream Akt Signaling and FOXO3a Regulation—To relate age-related changes in FOXO3a to upstream Akt signaling, VSMC were exposed to IGF-1, a growth factor known to phosphorylate Akt through activation of the phosphatidylinositol 3-kinase pathway and mediated by the IGF-1R (32, 33). Based on the proposed model for a FOXO3a shuttling mechanism, upon phosphorylation, P-Akt is quickly translocated from the cytoplasm to the nucleus (34), and nuclear phosphorylation of FOXO3a by P-Akt leads to relocalization of FOXO3a from the nucleus to the cytosol, thus resulting in a decrease in its transcriptional activity. To determine whether this shuttling mechanism contributed to down-regulation of MnSOD gene expression, VSMC were treated with IGF-1 (5 nM) for 1–8 h. Western blot analyses (Fig. 6A) indicated that the phosphorylation of Akt (at Ser473) in the nucleus was induced by ~2.1 times after exposure of VSMC from young rats to IGF-1 for 1 h, maintained for 6 h, and then declined to ~1.7 times control. The same
shows that P-FOXO3a was shuttled to the cytoplasm as early as 1 h after the addition of IGF-1 (by ~4.3-fold) and lasted for at least 8 h. In the same blot, we also observed the corresponding induction of IGF-1R after 1–4 h of IGF-1 stimulation of these cells. In the presence of IGF-1, MnSOD levels gradually declined by ~13% at 4 h and by ~59% at 6 h and persisted for 24 h (~41%), correlating with the inactivation of FOXO3a (Fig. 6B).

A reduction in FOXO3a function was also observed in a FOXO3A-SOD2 binding assay (Fig. 6C). FOXO3a binding activity was significantly reduced to ~20% at 1 h and to ~17% at 2 h, lasting for 8 h of IGF-1 treatment. Because it has been reported that DNA binding is interrupted by phosphorylation of the serine residue present in the forkhead DNA-binding domain (20), these data are consistent with phosphorylation of FOXO3a at Ser253, which is inside of the forkhead domain, leading to diminished DNA binding activity.

Effect of Age on Akt Activity—To determine whether there was an age-related difference in the activation of Akt responsible for the age-related effects on FOXO3a function and MnSOD expression in our model, two related experiments were performed to compare nuclear Akt activity in VSMC from young and old rats. First, the phosphorylation status at Ser473 and Thr308 in the nuclear protein from young and old VSMC was examined. Phosphorylation of Akt at Ser473, but not at Thr308, in the nuclear protein from VSMC from old rats was increased by ~2.2-fold when cells were grown in 5 mM glucose medium and by ~1.2 times when grown in 12.5 mM glucose medium compared with the young rats (Fig. 7A). Second, Akt kinase activity was tested using the GSK-3 fusion protein as a kinase substrate (Fig. 7B). Significantly higher Akt kinase activity was observed in VSMC from old versus young rats under control (5 mM glucose) and high physiologic (12.5 mM) glucose conditions (p < 0.01) as shown in Fig. 7C, consistent with the Akt phosphorylation results. These data

FIGURE 8. A, left panels, VSMC were grown in complete DMEM overnight, then pretreated with 10 μM AG-1024 for 30 min, and then changed to reduced medium containing 5 nM IGF-1 with or without 10 μM AG1024 for 4 h and compared with untreated controls. A representative Western blot sequentially probed for IGF-1R, P-Akt at Ser473, P-FOXO3a at Ser253, and β-actin is shown. Right panels, VSMC from old rats were grown in complete DMEM containing 5 mM glucose overnight. On the second day, they were treated with 10 μM AG-1024 or Me2SO vehicle only (control) in the medium containing 5 or 12.5 mM glucose for 3 days. Two representative Western blots, one showing the changes in P-Akt at Ser473 and P-FOXO3a at Ser253 and the other showing MnSOD, each with the corresponding β-actin loading control. B, VSMC from old rats grown on chamber slides were fixed and double immunocytochemically stained for P-Akt (green) and FOXO3a (red). The nuclei were co-stained with DAPI (purple). The slides were examined using a confocal laser scanning microscope LSM 510. The representative images were chosen from control (left panel) and AG1024-treated cells (right panel), respectively. C, a scheme summarizing the shuttling of P-Akt and P-FOXO3a between the nucleus and cytosol and its role in the transcriptional regulation of MnSOD. The scheme was derived from information in the literature and the data generated in these experiments. Briefly, GH stimulates IGF-1 binding to its receptor leading to phosphorylation of Akt. P-Akt, in turn, phosphorylates FOXO3a, preventing its binding to the promoter of the MnSOD gene and consequently reducing transcription. P-FOXO3a is translocated to the cytosol for degradation.

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confirm that there is indeed an age-related increase in Akt activity in VSMC from old rats.

Effect of IGF-1R Inhibition on Phosphorylation of Akt and FOXO3a—We have described the effect of IGF-1 treatment on P-Akt/P-FOXO3a shuttling. We also determined that there was an age-related increase in Akt activity in VSMC from old rats. To confirm these findings, an IGF-1R inhibitor, AG1024, was used to block the intracellular action of IGF-1. In the left panels of Fig. 8A, VSMC treated with 5 nM IGF-1 together with 10 μM AG1024 had clearly reduced IGF-1R β-chain protein compared with VSMC treated with IGF-1 only. As a consequence, 10 μM AG1024 attenuated the effect of 5 nM IGF-1 on P-Akt from ~204 to 129% of control and on P-FOXO3a from 291 to 148% of control, confirming its inhibition of both P-Akt and P-FOXO3a.

Because Akt phosphorylation and activity were increased in VSMC from old rats, we treated those VSMC with AG1024 as well. The right panels of Fig. 8A show that treatment of old VSMC with 10 μM AG1024 under control (5 mM glucose) conditions reduced P-Akt to ~55% of control, P-FOXO3a to ~66% of control, and in turn, increased MnSOD levels by ~168%. The dose of 10 μM AG1024 had less of an effect on VSMC grown in the 12.5 mM glucose medium.

To clarify the shuttling of P-Akt and P-FOXO3a in and out of the nucleus, the CSLM images double stained with P-Akt (Ser473) in green and FOXO3a (total) in red were used to show the relationship of their respective locations. In the left panel of Fig. 8B, a cell (chosen from old VSMC) showed a higher level of P-Akt in the nucleus, whereas most of the FOXO3a was located in the cytosol; in contrast, when P-Akt was reduced and primarily located in the perinuclear region (chosen from old VSMC treated with AG1024), FOXO3a was found in both the nucleus and the cytosol, as shown in right panel of Fig. 8B. Fig. 8C summarizes the above results in a scheme to illustrate the shuttling between P-Akt and P-FOXO3a and its role in the transcriptional regulation of MnSOD.

**DISCUSSION**

We demonstrate that there is a FOXO3A-binding site, located 1272 bp upstream of the coding region of the rat SOD2 promoter, by the DNA binding assay and the ChIP approach. This is the first demonstration that FOXO3a binds to the SOD2 promoter in the rat. Because DNA binding per se may either induce or inhibit transcription of specific genes as discussed by Dickinson et al. (35), we used the FOXO3a siRNA approach to inhibit FOXO3a and found reduced FOXO3a binding to the SOD2 promoter and concomitant reduction in MnSOD gene expression. This implies that FOXO3a does up-regulate SOD2.

Lower MnSOD protein and activity in VSMC from old rats was paralleled by a reduction in FOXO3a transcriptional activity, suggesting that FOXO3a may be the transcription factor responsible for the age-related down-regulation of MnSOD. Other potential transcription factors that could be involved in the regulation of MnSOD with advancing age have been excluded by the virtue of finding reciprocal activation of particular transcription factors with lower MnSOD levels. The present data from VSMC are consistent with previous reports of lower MnSOD protein and activity in the old (1, 9); our data have additionally shown that FOXO3a is responsible for the modulation of this age-related decline in MnSOD.

However, transcriptional expression of MnSOD is not only regulated by FOXO3a when exposed to more severe oxidative stress; in more complicated situations, other transcription factors may be involved in the regulation and overlap with the effects of advancing age. For example, unlike the FOXO3a response to serum starvation or the inhibition of growth factors, Storz et al. (36) showed that release of mitochondrial reactive oxygen species activated the NF-κB pathway, but not the FOXO3a pathway. We have also reported previously that VSMC from old animals had increased NF-κB activity under supraphysiologic levels of glucose or after the addition of TNF-α (37). Therefore NF-κB may be related to some of the increase in MnSOD protein in the current experiments under the same conditions.

These data in concert suggest that differential expression MnSOD with age might be regulated through overlapping of different pathways with one preferentially activated in response to specific stimuli, whereas the influence of FOXO3a would predominate under more physiologic conditions.

Activation of Akt has been widely implicated in vascular pathology, especially as related to angiotensin II-induced VSMC polyploidization and hypertrophy (38), which are also regarded as a biomarker of aging (39). Our data are the first to link advancing age with increased Akt activity in VSMC and consequent reduction in FOXO3a transcriptional activity leading to down-regulation of MnSOD.

Results from the present work show that activation of Akt by exposure of young cells to IGF-1 increases FOXO3a phosphorylation and decreases MnSOD levels, whereas inhibition of Akt activity by AG1024 in VSMC from old rats had the opposite effects. This work further supports the important interaction between the activation of Akt and the increase of P-FOXO3a with age and thereby provides a link to the reduction in MnSOD transcription.

The complexity of the interplay between Akt and FOXO transcription factors in the regulation of antioxidant defense with advancing age suggests multiple steps through which modulation may influence the ability of specific cells to respond to environmental stress, either metabolic or as a consequence of exogenous toxins or oxidants. A better understanding of the overlapping pathways of regulation may permit targeting of therapy to enhance antioxidant defense based on the mode of stress and the ages of the animals.

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