

constructed plasmid pMAL-c2x-rSMP30 was used to transform *E. coli* BL21 that had previously been transformed with pGro7 (Takara Bio, Tokyo) for expression of two chaperone proteins: GroEL and GroES. The resulting clone was used to express rat SMP30 as a fusion protein with MBP as follows. It was cultured at 37°C overnight in LB containing 0.2% glucose, 100 µg/ml ampicillin, and 20 µg/ml chloramphenicol. After the cell suspension was diluted 100-fold with the same culture medium, D-arabinose (4 mg/ml) was added for expression of the chaperone proteins and cultured for 1.6 h at 37°C. Then, the expression of MBP-SMP30 fusion protein was induced by adding isopropyl β-D-thiogalactoside to a final concentration of 0.3 mM and culturing for 3 h at 30°C. For control experiments, pMAL-c2x was used instead of pMAL-c2x-rSMP30, and a fusion protein of MBP with the α-fragment of β-gal was induced in the same way as above. The cells were washed with Dulbecco's phosphate buffered saline, resuspended in 20 mM Tris-HCl buffer (pH 7.4) containing 200 mM NaCl, and stored at -30°C until use.

Activity Staining and Western Blotting for Recombinant MBP-SMP30 Fusion Protein. Suspensions of the test and control cells were sonicated and centrifuged at $9,000 \times g$ for 30 min at 4°C. Each resulting supernatant (60 µg of protein) was electrophoresed on a 7% polyacrylamide gel by the method of Davis (25), with some modifications. Proteins in the gel were transferred onto a polyvinylidene fluoride membrane with 25 mM Tris/192 mM glycine as a transfer buffer. Subsequent staining for GNL activity involved immersing the membrane successively in 5 mM Tris-HCl buffer (pH 6.8) containing 1 mM DTT and 1 mM MnCl₂ (buffer A), then in a mixture of buffer A and 0.04% methyl red in 60% ethanol (9:1), and finally in buffer A containing 80 mM D-galactono-γ-lactone until the red color appeared.

For Western blotting, the supernatant (20 µg of protein) was electrophoresed in duplicate, and proteins were transferred onto a polyvinylidene fluoride membrane in the same way. The membrane was blocked overnight with a mixture of 2% BSA and 7% skim milk and cut into halves. One half was incubated with anti-MBP rabbit antibody (1:3,000 dilution; New England Biolabs), and the other half was incubated with anti-rat SMP30 rabbit antibody (1:3,000 dilution; Cosmo Bio, Tokyo) (3). Then, both half-membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:5,000 dilution; Cappel). MBP and SMP30 were visualized with an ECL (enhanced chemiluminescence) detection kit (Amersham Pharmacia Biosciences).

Measurement of AA. Plasma was mixed with nine volumes of 20% metaphosphate containing 1% SnCl₂, and the mixture was centrifuged at $10,000 \times g$ for 10 min at 4°C. Livers and kidneys were homogenized in 14 volumes of 5.4% metaphosphate, and the homogenate was centrifuged as above. For measurement of AA excreted into urine, a mouse was housed in a metabolic cage, and urine was collected in a bottle with 10% metaphosphate for 24 h. The amount of 10% metaphosphate had been adjusted to keep its concentration >5% after dilution with urine. The volume of the urine-metaphosphate mixture was recorded, and the mixture was centrifuged as above. All samples obtained after centrifugation were kept at -80°C until use. AA in samples was derivatized with dinitrophenylhydrazine and analyzed by HPLC with a Shodex-5SIL-4E column (4.6 × 250 mm; Showa Denko, Tokyo). The mobile phase was hexane/ethylacetate/acetic acid (5:4:1) at a flow rate of 1 ml/min, and the absorbance at 495 nm was recorded (26, 27). AA in mouse chow was determined by the same method after extraction into 15% metaphosphate.

Quantification of BMD and Body Fat Percentage. Subcranial total BMD and body fat percentage were determined by densitometry with the PIXImus2 imager (General Electric/Lunar, Madison, WI). Field calibration and calibration vs. the quality-control phantom were performed each day before imaging. Each mouse was positioned reproducibly in a prone position on the imaging tray and scanned three times. The coefficients of variance for BMD and body fat percentage were 0.9% and 2.2%, respectively, for *in vitro* measurements.

Statistical Analysis. The results are expressed as mean ± SEM. The probability of statistical differences between experimental groups was determined by Student's *t* test or ANOVA as appropriate. One and two-way ANOVAs were performed by using KALEIDAGRAPH software (Synergy Software, Reading, PA).

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- Fujita, T., Uchida, K. & Maruyama, N. (1992) *Biochim. Biophys. Acta* 1116, 122-128.
- Mori, T., Ishigami, A., Seyama, K., Onai, R., Kubo, S., Shimizu, K., Maruyama, N. & Fukuchi, Y. (2004) *Pathol. Int.* 54, 167-173.
- Ishigami, A., Fujita, T., Handa, S., Shirasawa, T., Koseki, H., Kitamura, T., Enomoto, N., Sato, N., Shimosawa, T. & Maruyama, N. (2002) *Am. J. Pathol.* 161, 1273-1281.
- Ishigami, A., Kondo, Y., Nanba, R., Ohsawa, T., Handa, S., Kubo, S., Akita, M. & Maruyama, N. (2004) *Biochem. Biophys. Res. Commun.* 315, 575-580.
- Kondo, Y., Ishigami, A., Kubo, S., Handa, S., Gomi, K., Hirokawa, K., Kajiyama, N., Chiba, T., Shimokado, K. & Maruyama, N. (2004) *FEBS Lett.* 570, 57-62.
- Kanagasundaram, V. & Scopes, R. (1992) *Biochim. Biophys. Acta* 1171, 198-200.
- Bublitz, C. & Lehninger, A. L. (1961) *Biochim. Biophys. Acta* 47, 288-297.
- Burns, J. J. (1960) in *Metabolic Pathway*, ed. Greenberg, D. M. (Academic, New York), Vol. 1, pp. 341-356.
- Nishikimi, M. & Yagi, K. (1996) in *Subcellular Biochemistry-Ascorbic Acid: Biochemistry and Biomedical Cell Biology*, ed. Harris, J. R. (Plenum, New York), Vol. 25, pp. 17-39.
- Nishikimi, M., Okamura, M. & Ohta, Y. (2003) in *Recent Research Developments in Biophysics and Biochemistry*, ed. Pandalai, S. G. (Research Signpost, Kerala, India), Vol. 3, Part 1, pp. 531-545.
- Grossman, S. H. & Axelrod, B. (1973) *J. Biol. Chem.* 248, 4846-4851.
- Shimazono, N. & Mano, Y. (1961) *Ann. N.Y. Acad. Sci.* 92, 91-104.
- Winkelman, J. & Lehninger, A. L. (1958) *J. Biol. Chem.* 233, 794-799.
- Kawada, M., Takiguchi, H., Kagawa, Y., Suzuki, K. & Shimazono, N. (1962) *J. Biochem. (Tokyo)* 51, 405-415.
- Rebouche, C. J. (1991) *Am. J. Clin. Nutr.* 54, 1147S-1152S.
- Ha, T. Y., Otsuka, M. & Arakawa, N. (1990) *J. Nutr. Sci. Vitaminol. (Tokyo)* 36, 227-234.
- Kono, K., Asai, K., Kuzuya, F. & Harada, T. (1990) in *Vitamin C and the Scurvy-Prone ODS Rat*, eds. Fujita, T., Fukase, M. & Konishi, T. (Elsevier, Amsterdam), pp. 147-155.
- Bernstein, S. E. (1966) in *Biology of the Laboratory Mouse*, ed. Green, E. L. (Dover, New York), 2nd Ed., pp. 337-350.
- Maeda, N., Hagihara, H., Nakata, Y., Hiller, S., Wilder, J. & Reddick, R. (2000) *Proc. Natl. Acad. Sci. USA* 97, 841-846.
- Kagawa, Y. & Takiguchi, H. (1962) *J. Biochem. (Tokyo)* 51, 197-203.
- Fujita, T., Shirasawa, T., Uchida, K. & Maruyama, N. (1992) *Biochim. Biophys. Acta* 1132, 297-305.
- Lindsay, R. M., Smith, W., Lee, W. K., Dominiczak, M. H. & Baird, J. D. (1997) *Clin. Chim. Acta* 263, 239-247.
- Hucho, F. & Wallenfels, K. (1972) *Biochim. Biophys. Acta* 276, 176-179.
- Nishikimi, M., Koshizaka, T., Mochizuki, H., Iwata, H., Makino, S., Hayashi, Y., Ozawa, T. & Yagi, K. (1988) *Biochem. Int.* 16, 615-621.
- Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* 121, 404-427.
- Kishida, E., Nishimoto, Y. & Kojo, S. (1992) *Anal. Chem.* 64, 1505-1507.
- Kodaka, K., Inagaki, S., Ujie, T., Ueno, T. & Suda, H. (1985) *Vitamin* 59, 451-455.



SMP30 deficiency causes increased oxidative stress in brain

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Abstract

Senescence marker protein 30 (SMP30), an important aging marker molecule, has been identified functionally as a calcium regulatory protein. Recent evidence showed its new assumed role as an effective anti-oxidative property. However, the role of SMP30 in the brain has not been explored. To delineate its role in the brain, we utilized SMP30 knock-out (SMP30 KO) mice in the current study. We focused on the oxidative status of the brain by examining selected oxidative markers in brains of SMP30 KO mice. Results showed that the generation of reactive species (RS) and NADPH oxidase activities were significantly elevated in SMP30 deficient brain. The increased oxidative status in these mice was further confirmed by increased oxidatively modified proteins such as dityrosine formation and carbonylation in the cortex of SMP30 KO mice. Moreover, SMP30 deficient brain showed the increased Mac-1 protein and myeloperoxidase (MPO) activity in the brain, supporting the putative anti-oxidative action of SMP30. Interestingly, the activities of major antioxidant enzymes, superoxide dismutase, catalase and reduced glutathione peroxidase in the brain were not affected by SMP30 depletion. Our results documented that brain SMP30 has a protective action against oxidative damage, without influencing antioxidant enzyme status.

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1. Introduction

SMP30 was discovered by Fujita who identified it as a senescence marker molecule that decreases an androgen-independently with aging (Fujita et al., 1992). SMP30 was later recognized to pose a Ca²⁺ binding capacity, for which named as regucalcin (Fujita et al., 1999).

Many recent evidences show that SMP30 has been demonstrated to play multifunctional roles as a regulatory protein in intracellular signaling processes, such as activation of Akt on apoptosis (Matsuyama et al., 2004) and the regulation of various Ca²⁺-dependent protein kinase and tyrosine kinase, protein phosphatases, nitric oxide (NO) synthase, and suppression of nuclear DNA and RNA syntheses (Izumi et al., 2003; Izumi and Yamaguchi, 2004).

The recently developed SMP30 knock-out (KO) mice have provided us the experimental paradigm to analyze the critical roles of SMP30. SMP30 KO mice were generated by disruption of the third exon of SMP30 gene on the X chromosome (Ishigami et al., 2002). Although these mice show about 20% lower in body weight and a shorter life span compared to the wild type (WT), they are fertile and apparently healthy (Maruyama et al., 2004). Many of the phenotypic changes mimic the premature aging processes. For example, SMP30 KO mice showed hepatic lipid droplets, abnormally enlarged mitochondria with indistinct cristae, and enlarged lysosomes (Ishigami et al., 2004). Likewise, deposition of lipofuscin as an aging marker in renal tubular epithelial cells was found in the kidney of SMP30 KO mice (Maruyama et al., 2004). Moreover, SMP30 KO mice have an increased susceptibility to apoptosis induced by TNF- α , Fas ligand, and calcium ionophore in hepatocytes (Ishigami et al., 2002).

Other reports indicate that SMP30 participates in controlling the antioxidant enzyme activity. SMP30 has an

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activating effect on superoxide dismutase (SOD) in liver (Fukaya and Yamaguchi, 2004) and heart cytosol (Ichikawa and Yamaguchi, 2004) of SMP30 transgenic mice. It has been suggested that the age-associated decrease of SMP30 is closely associated with oxidative stress. For instance, treatment of carbon tetrachloride as acute oxidative stress markedly suppresses the SMP30 expression in liver (Ishigami et al., 2001). In addition, SMP30 suppresses the generation of reactive species (RS) in hepatocytes (Feng et al., 2004), and decreased levels of SMP30 in aging process were correlated with the increased levels of RS generation in rat liver and kidney (Jung et al., 2004). It has also been reported that a significant reduction of SMP30 by lipopolysaccharide (LPS)-treated liver of 13-month-old rat (Jung et al., 2004). Since the hallmark of inflammatory process, in general, increased production of RS, it is rational to associated elevated oxidative stress with neuroinflammatory process (Floyd and Hensley, 2002; Emerit et al., 2004). Moreover, sustained accumulation of various oxidants causes chronic inflammation, which is characterized by the infiltration of leukocytes (Halliwell and Gutteridge, 1999). Therefore, in the present study, we attempted to define the role of SMP30 utilizing the knock-out model in relation to the oxidative status.

2. Materials and methods

2.1. Animal maintenance and tissue preparation

SMP30 deficient mice were previously prepared by the gene targeting (Ishigami et al., 2002). We used 12-months-old male mice in this study. Mice were maintained at 20–23 °C on a 12 h light/12 h dark cycles in controlled environment and fed ad libitum using the procedures approved by The Animal Care and Use Committee of Tokyo Metropolitan Institute of Gerontology.

Cortical tissues were prepared in ice-cold homogenization solution (50 mM potassium phosphate buffer containing 1 mM EDTA, 1 mM para-nitrobenzamide, 1 μ M pepstatin, pH 7.2) and disrupted for 20 s with an ultrasonic device. The samples were centrifuged at 12,000 rpm at 4 °C for 30 min. The supernatant was regarded as a whole fraction, and the protein concentration was determined by the method of bicinchoninic acid (BCA) solution.

2.2. Reagents

2',7'-Dichlorofluorescein diacetate (DCFDA) was obtained from Molecular Probes, Inc. (Eugene, OR, USA). Western blot detection reagent was obtained from Amersham (Amersham, Bucks, UK). Polyvinylidene difluoride (PVDF) membranes were obtained from Millipore Corporation (Bedford, MA, USA). All antibodies and other chemical reagents were purchased from Sigma (St. Louis, MO, USA).

2.3. Assessment of oxidative stress

RS generation was measured as previously described, utilizing a fluorescence probe (Thomas et al., 1992). Briefly, 25 μ M DCFDA was added to homogenates for 250 μ l of final volume. Changes in fluorescence intensity were measured at 10, 20, 30 and 40 min on a Fluorescence Plate Reader (TECAN, Austria), with excitation and emission wavelengths set at 485 and 530 nm, respectively. The changes of fluorescence intensity were normalized by time (min) and protein concentration.

2.4. Enzyme assays

2.4.1. SOD activity assay

SOD activity was measured using the modified xanthine-oxidase-cytochrome *c* method as described by MaCord and Fridovich (1969). The final concentrations of mixture in e-tube were 50 mM potassium phosphate (pH 7.8), 10 mM xanthine, 0.1% deoxycholate, 50 μ M potassium cyanide, 0.1 mM ferricytochrome-*c*, and 0.5 U xanthine oxidase. After well vortexed, the mixture was incubated in heat block at 22 °C for 2 min. Once heated, the reaction was initiated by addition of homogenates (0.03–0.05 mg) into the mixture. The decrease in absorbance at 550 nm was measured for 3 min.

2.4.2. Catalase activity assay

Catalase activity was assayed according to the method (Beers and Sizer, 1953). The final concentrations in the cuvettes were 50 mM potassium phosphate (pH 7.0), 30 mM H₂O₂ and the samples (0.03–0.05 mg). The decrease in absorbance at 240 nm after the addition of the samples was followed spectrophotometrically.

2.4.3. Glutathione peroxidase (GSH-Px) activity assay

GSH-Px activity was assayed with a coupled enzyme system in which glutathione (GSSG) reduction was coupled to NADPH oxidation by glutathione reductase (Lawrence and Burk, 1976). The assay mixture contained 50 mM potassium phosphate (pH 7.5), 1 mM EDTA, 1 mM NaN₃, 1 mM reduced glutathione (GSH), 0.2 mM NADPH, 1 U glutathione reductase and tissue samples (0.05–0.2 mg). After 5 min preincubation (20–25 °C), the reaction was initiated by the addition of 0.25 mM H₂O₂. The decrease in the absorbance at 340 nm was followed spectrophotometrically.

2.4.4. NADPH oxidase activity assay

NADPH consumption was assayed spectrophotometrically at 340 nm (Zhang et al., 2000). The oxidized nucleotides NADP⁺ do not show any absorbance at 340 nm (Souza et al., 2002). The reaction observed contained the sample (0.05–0.2 mg), diluted to a final 50 mM potassium phosphate (pH 7.5). NADPH (final 1 mM) was added, just before the measurements were started, and followed for 5 min. The decreased absorbance at 340 nm was measured and applied as NADPH oxidase activity.

2.4.5. MPO activity assay

This assay was designed to measure the production of tetramethylbenzidine (TMB) oxidation by MPO/H₂O₂. Samples were mixed with reacting reagent (15 mM TMB, 100 mM sodium acetate, 60 mM hydrogen peroxide). The reaction was monitored at 650 nm for 30 min on a Genios TECAN Spectrophotometer (Austria). Data were expressed mU/mg, protein. One unit of activity is defined as the amount of enzyme that will utilize 1 μ mol of hydrogen peroxide/min. In this assay, one milli unit of MPO will cause absorbance change of 0.0114 min⁻¹ at a 650 nm.

2.5. Western blot analysis

Western blotting was carried out with whole fractions of the cortical tissues. The samples were boiled for 5 min with a gel-loading buffer (pH 6.8): 0.125 M Tris-HCl, 4% sodium dodecyl sulfate, 20% glycerol, 10% 2-mercaptoethanol, and 0.2% bromophenol blue in a ratio of 1:1. Total protein equivalents for each sample were separated on an SDS-polyacrylamide mini gel using 10 or 15% acrylamide gels at 100 V and transferred to a PVDF membrane at 70 V for 1 h. The membrane was immediately placed into a blocking solution (5% skim milk) at room temperature for 30 min. The membrane was incubated with a diluted primary antibody; SMP30 (1:5000), dityrosine (IC3; 200 ng/ml) (Kato et al., 1998), dinitrophenyl (DNP; 1:1000), SOD (1:2000), catalase (1:1000), Mac-1 (1:1000) and β -tubulin (1:4000) in TBS-Tween buffer at room temperature for 3 h. The membrane was followed by incubation with secondary antibody; polyclonal anti-goat, rabbit, mouse, or rat antibody (1:40,000) in Tween buffer at room temperature for 1 h. Horseradish-conjugate secondary antibody labeling was detected by enhanced chemiluminescence (ECL) Western blotting reagents and analysis (Amersham Bioscience, NJ) following the manufacturer's instructions. Pre-stained blue protein markers were used for molecular weight determination.

2.6. Pathological analysis

Twelve-months-old male WT and SMP30 KO mice were euthanized and whole brains were removed. Frozen brains were sectioned coronally at 20 μ m on a cryostat and were thaw-mounted onto Superfrost Plus™ slides (Fisher, Chicago, IL). Slides were maintained at -20°C until they were processed for histology. Coronal frozen sections through hippocampus were prepared and stained with cresyl violet (Nissl) for evaluation of neuronal morphology (Wu et al., 2005).

2.7. Statistical analysis

The statistical significance of the differences between WT and SMP30 KO mice was determined by ANOVA with PLSD. Values of $p < 0.05$ were considered statistically significant. Analyses were performed using Statview software®.

3. Results

3.1. Increased RS levels and NADPH oxidase activities in SMP30 KO mice brain

To evaluate the oxidative stress in SMP30 KO mice, we measured total RS levels in the cortex of WT and SMP30 KO mice using DCFDA method (Thomas et al., 1992). Total RS levels in SMP30 KO brain were significantly increased compared to WT mice (Fig. 1A; $p < 0.001$). NADPH oxidase is well known as a major enzyme to generate superoxide anion (Clark et al., 2004). Therefore, we measured NADPH oxidase activities to determine whether RS source enzyme activity was responsible for the elevated total RS levels in the mice. NADPH

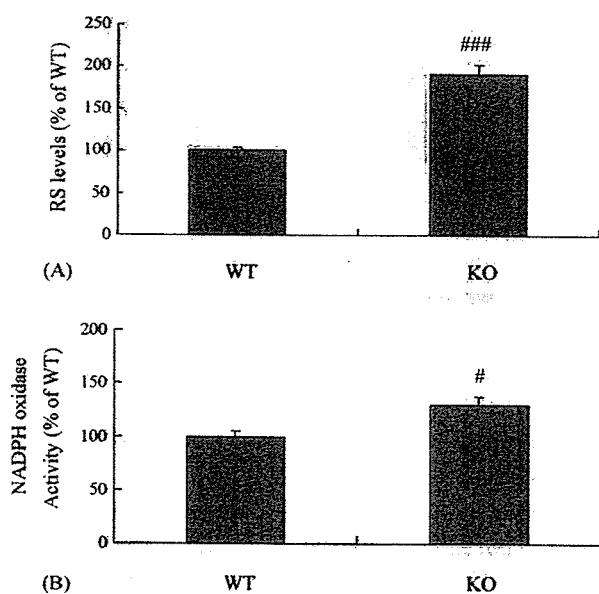


Fig. 1. Levels of reactive species (RS) and activities of NADPH oxidase were increased in SMP30 KO brain. (A) Total RS levels were detected by DCFDA method in cortex of WT and SMP30 KO mice. Levels of RS were significantly increased in SMP30 deficient cortex compared to WT. (B) NADPH oxidase activities were measured by NADPH consumption as substrate at 340 nm spectrophotometer. SMP30 depletion resulted in the elevated activities of NADPH oxidase in the cortex. Each value is the mean \pm S.E. of four to five mice in two times experiment with triplicates. As significance, results of one-factor ANOVA: # $p < 0.05$, ### $p < 0.001$ vs. WT.

oxidase activities were consistently elevated with increased RS levels in SMP30 KO brain (Fig. 1B; $p < 0.05$).

3.2. Antioxidant enzyme status

To assess whether the increased oxidative stress is caused by the dysfunction of oxidative defense system in the SMP30 KO brain, we measured the activities and levels of several antioxidative enzymes, including SOD, catalase, and GSH-Px in cortex. However, no marked differences in activities of SOD, catalase, and GSH-Px were detected between WT and SMP30 KO cortex (Fig. 2A). The protein levels of either SOD or catalase were not significantly different between WT and SMP30 KO brain (Fig. 2B). This result demonstrated that antioxidant enzyme status in brain was not affected by SMP30 depletion.

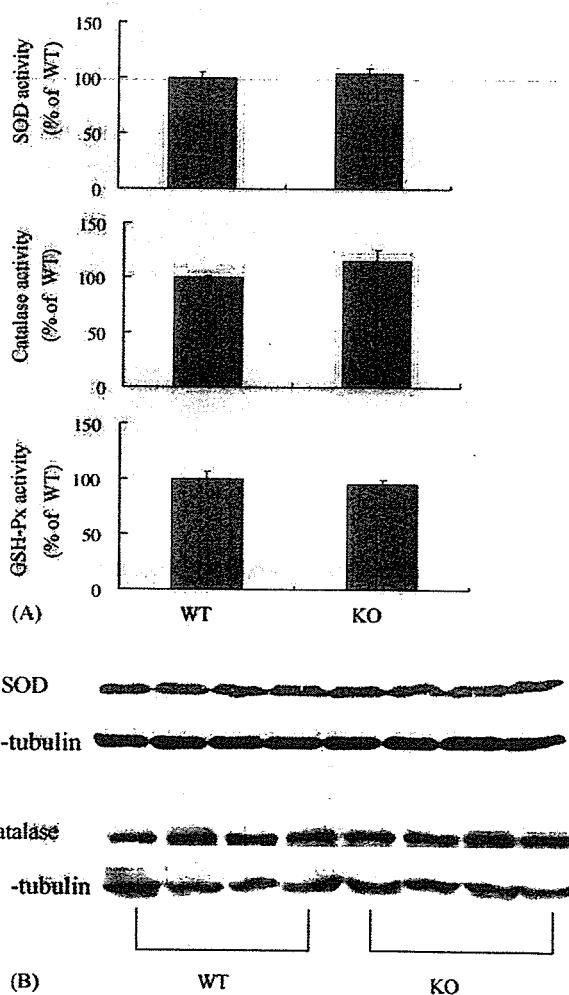


Fig. 2. The antioxidative enzyme status was not altered by SMP30 depletion in the cortex. Activities (A) and protein levels (B) of antioxidant enzymes were examined in the cortical tissues of WT and KO mice. No significant changes were detected in activities of superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSH-Px) between WT and KO mice. (B) Immunoblot analysis showed that protein levels of SOD and catalase were not altered by SMP30 depletion. Levels of β -tubulin used to normalize the levels of protein loaded. Values are expressed as a percentage of WT mice brain and represent means \pm S.E. of four mice.

3.3. Enhancement of protein oxidation in SMP30 KO mice brain

We further investigated to evaluate protein oxidation in the cortical tissue from WT and SMP30 KO mice. We examined the dityrosine formation and carbonylation (DNP; dinitrophenyl), because both dityrosine formation (Giulivi et al., 2003) and carbonylation (Liu et al., 2003) was used as markers of protein oxidation. We were able to confirm that SMP30 protein expression was completely depleted in the cortex of SMP30 KO mice (Fig. 3A). Levels of dityrosine and DNP (Fig. 3B; $p < 0.01$ and $p < 0.05$, respectively) were elevated in the cortex

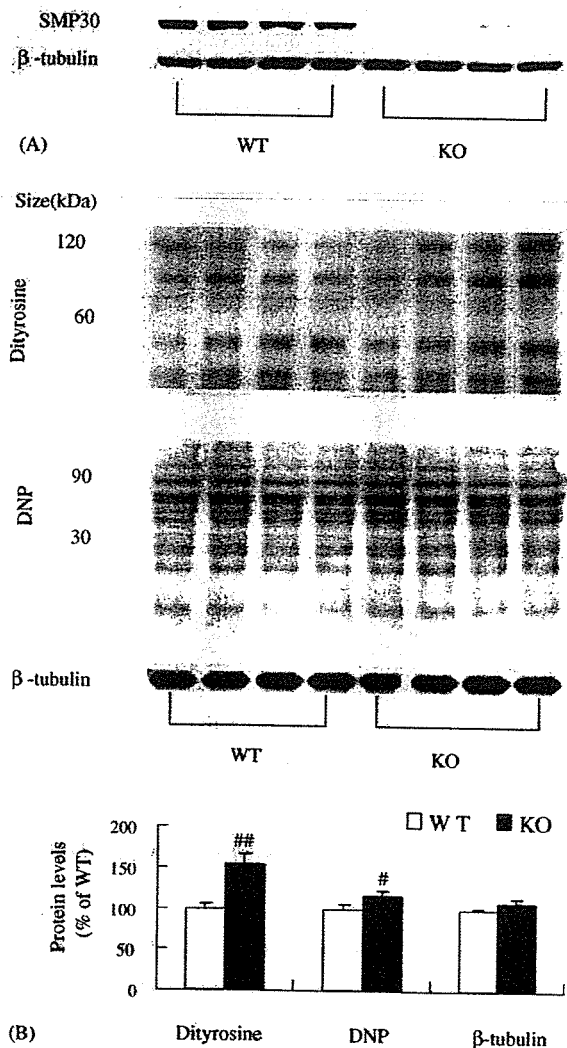


Fig. 3. Protein oxidation was increased in cortex of SMP30 KO mice. (A) Immunoblot analysis showing SMP30 protein levels in WT and KO. (B) Dityrosine formation and carbonylation (DNP) was examined in the mice brain. The cortical homogenates were subjected to immunoblot analysis using anti-dityrosine and anti-DNP. Whole proteins were screened and around size between 60 and 120 kDa of tyrosylated proteins and between 60 and 90 kDa of carbonylated proteins were markedly elevated in SMP30 KO brain compared to WT. Levels of β -tubulin used as protein loading control. Values are expressed protein levels as a percentage of WT mice brain and represent means \pm S.E. of four mice. As significance, results of one-factor ANOVA: # $p < 0.05$, ## $p < 0.01$ vs. WT.

of SMP30 KO mice compared to WT mice, indicating that more protein oxidation occurred in SMP30 KO brain. These results suggest that SMP30 deficient brains might be functionally affected by the increased protein oxidation.

3.4. Activated leukocytes in SMP30 KO mice brain

It seems clear that SMP30 KO mice show elevated RS and protein oxidation in the brain, without alteration of antioxidant enzyme status. Since it has been reported that chronic inflammation can be also caused by RS, we hypothesized that activated immune cells were infiltrated into the SMP30 KO brain, which cause unusual release of oxidants. To valid this hypothesis, myeloperoxidase (MPO) activity, a reliable marker for leukocyte recruitment and activation (Morken et al., 2002; Squadrito et al., 2000), was measured in the cortex WT and SMP30 KO mice. The markedly increased MPO activities in SMP30 KO mice indicated the infiltration of activated leukocytes (Fig. 4A). To further confirm our assumption, the level of Mac-1 was determined in the brain homogenates, because Mac-1 is regarded as the key mediator responsible for the migration of neutrophil (Clark et al., 1996). The levels of Mac-1 in SMP30 KO brain were as high as 1.5-fold ($p < 0.01$) of that in WT (Fig. 4B). These results clearly demonstrated the accumulation of leukocytes in the brain tissues taken from SMP30 KO mice, which may be responsible for the elevated RS levels.

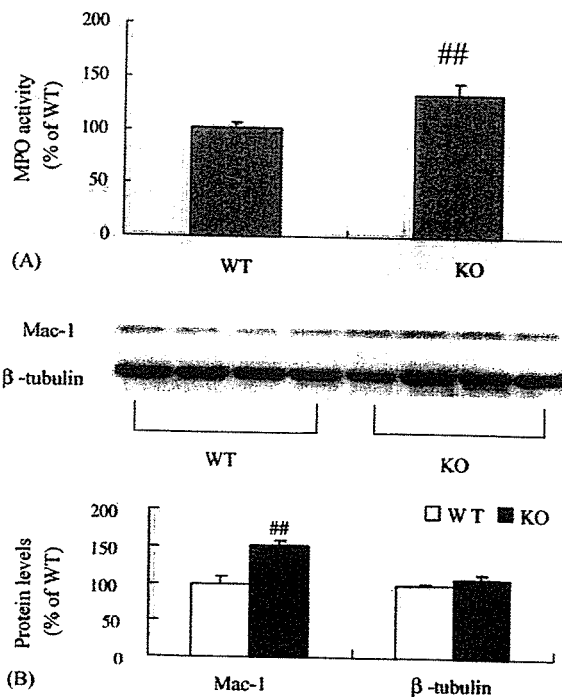


Fig. 4. Infiltration and activation of leukocyte was increased in SMP30 KO brain. (A) MPO activities were measured as an infiltrated leukocyte marker by specific assay in the mice. MPO activities significantly increased in SMP30 KO brain. Values are the mean \pm S.E. of four mice. As significance, results of one-factor ANOVA: ## $p < 0.01$ vs. WT. (B) The protein levels of Mac-1 were quantified arbitrarily as an activated leukocyte marker by densitometry. Values are expressed protein levels as a percentage of WT mice. As significance, results of one-factor ANOVA: ## $p < 0.01$ vs. WT.

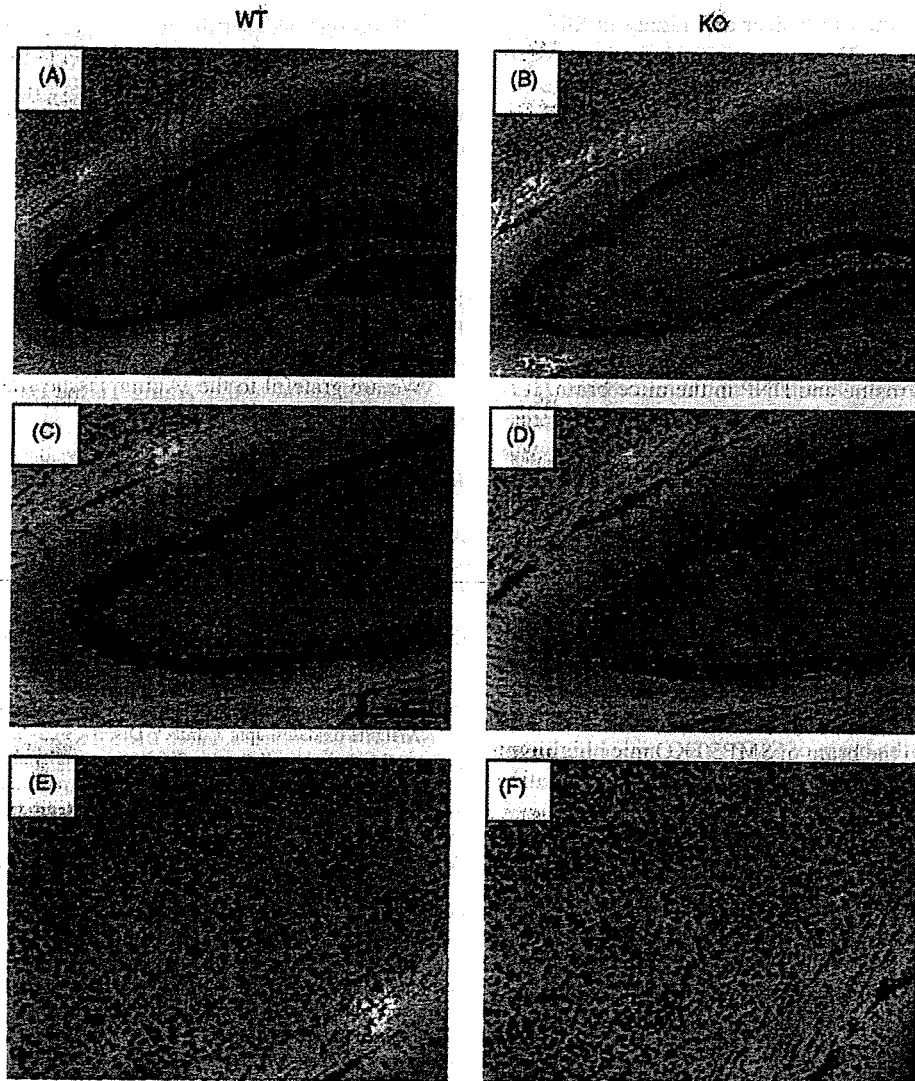


Fig. 5. SMP30 KO mice did not show any neuronal pathology in the brain compared to WT mice. Coronal brain sections of hippocampus were stained with cresyl violet (Nissl) to evaluate neuronal loss and damage. There were no neuronal losses or damages in both WT mice (A, C, E) and SMP30 KO mice (B, D, F). Nissl images of CA1 and CA3 region (C, D) and the cortex (E, F). Scale bar: 100 μm (A, B), 50 μm (C–F).

3.5. Histopathology of SMP30 KO mice brain

We examined the sectioned brain tissues of SMP30 KO mice for the possible pathological changes that might cause the increased oxidative stress and leukocyte infiltration. Nissl stained slides were evaluated for any neuronal damages. The results found that there were no neuronal loss or damage in coronal brain sections of both WT (Fig. 5A, C and E) and SMP30 KO mice (Fig. 5B, D and F). Based on these data, we concluded that increased oxidative status and neutrophil activation in SMP30 KO brains we observed were not likely due to the secondary effects of the pathological changes of the brain.

4. Discussion

Most studies on SMP30 have been focused on the kidney and liver, because expression levels of SMP30 are particularly high

in those organs. However, studies on the SMP30's tissue or organ specificity with relatively low levels have not been well studied. The brain is one of those organs with low levels of SMP30, but do express SMP30. The brain is known to be susceptible to damaging effects of oxidative stress due to their high oxygen consumption and relatively low antioxidant capacity (Anderson, 2004). The current study is the first report on the characterization of brain SMP30 utilizing SMP30 KO mice in relation with oxidative status.

We assessed the oxidative status of SMP30 KO brain by measuring several key markers for oxidative stress. Included are: total RS levels for biochemical marker, NADPH oxidase for an enzymatic marker, and MPO activities (as a physiological marker), and found that all are significantly increased in the brain of SMP30 KO mice. Consistent with the oxidatively stressed SMP30 KO brain, there is the increased leukocyte activity as shown in Fig. 4. These observations are consistent

with our preliminary studies with liver and kidney in SMP30 KO mice (data not shown). However, our oxidatively stressed brain was not accompanied with any compensatory changes in activities or protein levels of anti-oxidative defense enzymes in SMP30 KO brain. These results indicate that SMP30 has suppressive action against oxidative stress in the brain without modulating antioxidant enzyme status. This finding raises the possibility of the organ specificity of SMP30 in response to oxidative stress in which case, the SMP30 KO brain seems to have an impaired capacity to induce, i.e. synthesize those scavenging enzymes in response to oxidative stress.

Elevated RS in SMP30 KO brain was verified by detecting protein oxidation; dityrosine and DNP in the mice brain. It is likely that the lack of SMP30 with increased oxidative stress is responsible for the elevated protein oxidation. It was recently reported that protein oxidation by increased oxidative stress has been identified in several neurodegenerative disorders and aging processes. For example, the occurrence of dityrosine in proteins has a wide range of implication in atherosclerosis, inflammation, neurodegenerative disease, and aging (Leeuwenburgh et al., 1999; Hensley et al., 1998). Likewise, toxic carbonyl species have been associated with the pathophysiology of Alzheimer's disease (AD) (Liu et al., 2003). Thus, our findings on the increased dityrosine and carbonylation levels in the brain of SMP30 KO mice highlight a putative important role in the brain as a potent anti-oxidative function.

Other data revealed from the current study support our contention about SMP30's role in the brain. We found that protein levels of Mac-1 as marker of activated leukocyte were increased in SMP30 KO mice. Adhesion and migration of leukocytes is regulated by specific receptor–ligand interactions between intracellular adhesion molecule-1 (ICAM-1) on endothelial cells and a group of Mac-1 glycoproteins on leukocytes (Chao et al., 2000). To probe the infiltration of migrated leukocyte, we measured MPO activity in the mice, because identification of MPO can be used as neutrophil infiltration. We also found that MPO activities are consistent with increased levels of Mac-1 protein in SMP30 KO mice brain. These results indicate that the increased MPO activities are further confirming the status of oxidative stress in SMP30 KO mice brain.

One could suspect that the infiltration of neutrophil and increased oxidative stress were associated with the pathological processes in these KO mouse brain. However, our gross microscopic analysis of the brain shown in Fig. 5 revealed no discernable differences in sizes and shapes of hippocampus and cortex in SMP30 KO mice compared to wild type mice. In addition, histopathological analysis with Nissl staining indicates that there were no neuronal losses or damages in SMP30 KO mice compared to WT mice. Therefore, it is not likely that increased oxidative stress and leukocyte infiltration in SMP30 KO brains we observed in these KO mice are from the secondary effects of brain lesion. However, it is still probably true that SMP30 KO mice are more susceptible to pathological insults and vulnerable to various stresses due to the weakened anti-oxidative defenses.

Putting together, our findings suggest that SMP30 plays an important neuroprotective role in suppressing oxidative stress by modulating activation and infiltration of leukocytes, but without compensatory increases in endogenous oxidative defense enzymes.

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References

- Anderson, J.K., 2004. Oxidative stress in neurodegeneration: cause or consequence? *Nature* 10, S18–S25.
- Beers Jr., R.F., Sizer, I.W., 1953. Catalase assay with special reference to manometric methods. *Science* 117, 710–712.
- Clark, R.S., Carlos, T.M., Schiding, J.K., 1996. Antibodies against Mac-1 attenuate neutrophil accumulation after traumatic brain injury in rats. *J. Neurotrauma* 13, 333–341.
- Clark, R.A., Epperson, T.K., Valente, A., 2004. Mechanisms of activation of NADPH oxidases. *Jpn. J. Infect. Dis.* 57, S22–S23.
- Chao, G., Hoff, J.T., Keep, R., 2000. Acute inflammatory reaction following experimental intracerebral hemorrhage in rat. *Brain Res.* 871, 57–65.
- Emerit, J., Edeas, M., Bricare, F., 2004. Neurodegenerative diseases and oxidative stress. *Biomed. Pharmacother.* 58, 39–46.
- Feng, D., Kondo, Y., Ishigami, A., 2004. Senescence marker protein 30 as a novel antiaging molecule. *Ann. N.Y. Acad. Sci.* 1019, 360–364.
- Fujita, T., Uchida, K., Maruyama, N., 1992. Purification of senescence marker protein-30 (SMP30) and its independent decrease with age in the rat liver. *Biochim. Biophys. Acta* 1116, 122–128.
- Fujita, T., Shirasawa, T., Maruyama, N., 1999. Expression and structure of senescence marker protein-30(SMP30) and its biological significance. *Mech. Age. Dev.* 107, 271–280.
- Fukaya, Y., Yamaguchi, M., 2004. Regucalcin increases superoxide dismutase activity in rat liver cytosol. *Biol. Pharm. Bull.* 27, 1444–1446.
- Floyd, R.A., Hensley, K., 2002. Oxidative stress in brain aging implications for therapeutics of neurodegenerative disease. *Neurobiol. Age.* 23, 795–807.
- Giulivi, C., Traaseth, N.J., Davies, K.J.A., 2003. Tyrosine oxidation products: analysis and biological relevance. *Amino Acids* 25, 227–232.
- Halliwell, B., Gutteridge, J., 1999. *Free Radical in Biology and Medicine*, third ed. Oxford Press, Oxford, UK.
- Hensley, K., Maidt, M.L., Yu, Z.Q., 1998. Electrochemical analysis of protein nitro-tyrosine and dityrosine in the Alzheimer brain indicates region-specific accumulation. *J. Neurosci.* 18, 8126–8132.
- Izumi, T., Tsurusaki, Y., Yamaguchi, M., 2003. Suppressive effect of endogenous regucalcin on nitric oxide synthase activity in cloned rat hepatoma H4-II-E cells overexpressing regucalcin. *J. Cell. Biochem.* 89, 800–807.
- Izumi, T., Yamaguchi, M., 2004. Overexpression of regucalcin suppresses cell death in cloned rat hepatoma H4-II-E cells induced by tumor necrosis factor-alpha or thapsigargin. *J. Cell. Biochem.* 92, 296–306.
- Ishigami, A., Fujita, T., Simbula, G., et al., 2001. Regulatory effects of senescence marker protein 30 on the proliferation of hepatocytes. *Pathol. Int.* 51, 491–497.
- Ishigami, A., Fujita, T., Handa, S., et al., 2002. Senescence marker protein-30 knockout mouse liver is highly susceptible to TNF- α - and Fas-mediated apoptosis. *Am. J. Pathol.* 161, 1273–1281.
- Ishigami, A., Kondo, Y., Nanba, R., et al., 2004. SMP30 deficiency in mice causes an accumulation of neutral lipids and phospholipids in the liver and shortens the life span. *Biochem. Biophys. Res. Commun.* 315, 575–580.

- Ichikawa, E., Yamaguchi, M., 2004. Regucalcin increase superoxide dismutase activity in the heart cytosol of normal and regucalcin transgenic rats. *Int. J. Mol. Med.* 14, 691–695.
- Jung, K.J., Ishigami, A., Maruyama, N., et al., 2004. Modulation of gene expression of SMP30 by LPS and calorie restriction during aging process. *Exp. Gerontol.* 39, 1169–1177.
- Kato, Y., Maruyama, W., Naoi, M., 1998. Immunohistochemical detection of dityrosine in lipofuscin pigments in the aged human brain. *FEBS Lett.* 439, 231–234.
- Liu, Q., Raina, A.K., Smith, M.A., 2003. Hydroxynonenal, toxic carbonyls, and Alzheimer disease. *Mol. Aspects Med.* 24, 305–313.
- Leeuwenburgh, C., Hansen, P.A., Holoszy, J.O., 1999. Oxidized amino acids in the urine of aging rats. *Am. J. Physiol.* 45, R128–R135.
- Lawrence, R.A., Burk, R.F., 1976. Glutathione peroxidase activity in selenium deficient rat liver. *Biochem. Biophys. Res. Commun.* 71, 952–958.
- MacCord, J.M., Fridovich, I., 1969. The utility of superoxide dismutase in studying free radical reactions. I. Radicals generated by the interaction of sulfite, dimethyl sulfoxide, and oxygen. *J. Biol. Chem.* 244, 6056–6063.
- Morken, J.J., Warren, K.U., Xie, Y., 2002. Epinephrine as a mediator of pulmonary neutrophil sequestration. *Shock* 18, 46–50.
- Maruyama, N., Ishigami, A., Kuramoto, M., et al., 2004. Senescence marker protein-30 knockout mouse as an aging model. *Ann. N.Y. Acad. Sci.* 1019, 383–387.
- Matsuyama, S., Kitamura, T., Enomoto, N., et al., 2004. Senescence marker protein-30 regulates Akt activity and contributes to cell survival in Hep G2 cells. *Biochem. Biophys. Res. Commun.* 321, 386–390.
- Thomas, P., Herbert, D.G., James, P.K., 1992. Production of reactive oxygen by mitochondrial from normoxic and hypoxic rat heart tissue. *Free Radic. Biol. Med.* 13, 289–297.
- Souza, H.P., Liu, X., Zweier, J.L., 2002. Quantitation of superoxide generation and substrate utilization by vascular NAD(P)H oxidase. *Am. J. Physiol. Heart Circ. Physiol.* 282, H466–H474.
- Squadrito, F., Alrtavilla, D., Squadrito, G., 2000. Tacrolimus limits polymorphonuclear leukocyte accumulation and protects against myocardial ischemia-reperfusion injury. *J. Mol. Cell Cardiol.* 32, 429–440.
- Wu, G., Lu, Z., Wang, J., 2005. Enhanced susceptibility to kainate induced seizures, neuronal apoptosis and death in mice lacking gangliotetraose ganglioside: protection with LIGA 20 a membrane permeant analog of GM1. *J. Neurosci.* 25, 11014–11022.
- Zhang, Z., Yu, J., Stanton, R., 2000. A method for determination of pyridine nucleotide using a single extract. *Anal. Biochem.* 285, 163–167.

Senescence Marker Protein-30 Protects Mice Lungs from Oxidative Stress, Aging, and Smoking

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Rationale: Senescence marker protein-30 (SMP30) is a multifunctional protein providing protection to cellular functions from age-associated deterioration. We previously reported that SMP30 knockout (SMP30Y^{-/-}) mice are capable of being novel models for senile lung with age-related airspace enlargement and enhanced susceptibility to harmful stimuli.

Objectives: Aging and smoking are considered as major contributing factors for the development of pulmonary emphysema. We evaluated whether SMP30Y^{-/-} mice are susceptible to oxidative stress associated with aging and smoking.

Methods: Age-related changes of protein carbonyls in lung tissues from the wild-type (SMP30Y^{+/+}) and SMP30Y^{-/-} mice were evaluated. Both strains were exposed to cigarette smoke for 8 wk. Histopathologic and morphologic evaluations of the lungs, protein carbonyls and malondialdehyde in the lung tissues, total glutathione content in the bronchoalveolar lavage fluid, and degree of apoptosis of lung cells were determined.

Measurements and Main Results: In the lungs of SMP30Y^{-/-} mice, protein carbonyls tended to increase with aging and were significantly higher than the age-matched SMP30Y^{+/+} mice. Cigarette smoke exposure generated marked airspace enlargement (23.3% increase of the mean linear intercepts) with significant parenchymal destruction in the SMP30Y^{-/-} mice but not in the SMP30Y^{+/+} mice (5.4%). The protein carbonyls, malondialdehyde, total glutathione, and apoptosis of lung cells were significantly increased after 8-wk exposure to cigarette smoke in the SMP30Y^{-/-} mice.

Conclusions: Our results suggest that SMP30 protects mice lungs from oxidative stress associated with aging and smoking. The SMP30Y^{-/-} mice could be useful animal models for investigating age-related lung diseases, including cigarette smoke-induced pulmonary emphysema.

Keywords: aging; oxidative stress; pulmonary emphysema; senescence marker protein-30; smoking

Senescence marker protein-30 (SMP30), a 34-kD protein originally identified from the rat liver, is a novel molecule that decreases with age in an androgen-independent manner, suggesting

its possible role in age-related physiologic and pathologic conditions (1–3). We demonstrated that SMP30 is widely expressed in vertebrates and that the amino acid alignment is highly conserved (2, 4). In mice, SMP30 transcripts are detected in various organs, including the liver, kidney, cerebrum, testis, and lung (5). In humans, the SMP30 gene is located in the p11.3–q11.2 segment of the X chromosome (6). To clarify the physiologic role of SMP30 in age-associated organ disorders, the SMP30 knockout (SMP30Y^{-/-}) mouse was developed with gene targeting from C57BL/6 mice (7). We revealed that SMP30Y^{-/-} mice are viable and fertile but have reduced weight gain and shorter life span than the wild-type (SMP30Y^{+/+}) mice (8). Hepatocytes isolated from SMP30Y^{-/-} mice were shown to be highly susceptible to tumor necrosis factor- α and Fas-mediated apoptosis (7). Furthermore, exogenously induced SMP30 was shown to increase Ca²⁺ efflux by activating the calmodulin-dependent Ca²⁺ pump in HepG2 cells and thus to confer resistance to cell death caused by an increase in intracellular Ca²⁺ concentration (9). These findings suggest that SMP30 protects cells and organs from various injuries during the course of life.

There are many studies concerned with the relationship between aging and oxidative stress. Moderate oxidative stress may gradually develop with age because plasma levels of lipoperoxidation products and antioxidant enzyme activities in red blood cells increase with aging, whereas plasma levels of nutritional antioxidants decrease (10). The lungs are persistently exposed to oxidants generated endogenously from phagocytes and other cell types or exogenously from air pollutants or cigarette smoke (11). Pulmonary emphysema is an age-related lung disease that occurs after a prolonged period of cigarette smoking. Because cigarette smoke contains around 10¹⁷ oxidant molecules per puff and generates oxidant/antioxidant imbalance in the lungs, oxidative stress is postulated to play an important role in the pathogenesis of emphysema (11). In patients with chronic obstructive pulmonary disease, biomarkers of oxidative stress, such as protein carbonyls and lipid peroxidation products, are reported to be elevated in the lungs (12) and respiratory muscles (13).

We previously reported that SMP30Y^{-/-} mice develop peripheral airspace enlargement without alveolar destruction and may thus be a novel model for senile lung (5). We hypothesized that SMP30Y^{-/-} mice may be susceptible to oxidative stress with aging. Furthermore, SMP30Y^{-/-} mice may be vulnerable to cigarette smoke exposure and generate pulmonary emphysema. In this study, we investigated the age-related changes of protein carbonyls in the lungs of SMP30Y^{-/-} and SMP30Y^{+/+} mice and pathologically evaluated the effect of cigarette smoke exposure to the lungs and biomarkers of oxidative stress.

METHODS

Animals

We used SMP30Y^{-/-} and SMP30Y^{+/+} mice to investigate the age-related changes of mice lungs with 1-, 3-, 6-, and 12-mo-old mice (n = 5 for each group). We used 3-mo-old mice (n = 6 for each group) for the smoking experiment. Animal experimentation was approved by the

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Animal Care and Use Committee of Tokyo Metropolitan Institute of Gerontology and by Juntendo University, School of Medicine.

Preparation and Morphologic Evaluation of the Lungs

Mice were killed, and the lungs were processed as described previously (14). The lungs were lavaged with 0.5 ml of phosphate-buffered saline through an intratracheal cannula four times. The bronchoalveolar lavage fluid (BALF) was pooled, and total cell counts and cell populations in each BALF specimen were determined. The BALF was centrifuged, and the supernatant was collected for biochemical analysis.

Airspace size was assessed by determining the mean linear intercepts (MLI) according to the method previously described (15). The destructive index (DI) was determined to evaluate the severity of alveolar wall destruction (16). A DI value greater than 10% was considered to have significant destruction of the lung parenchyma (17).

Determination of Protein Carbonyls in the Lungs

The measurement of protein carbonyls in the supernatant of lung homogenates was conducted with a spectrophotometer as described previously (18–20). To confirm the specificity of the reaction of the proteins with 2,4-dinitrophenylhydrazine (DNPH), the lung extracts of SMP30Y/– mice were pretreated with sodium borohydride to reduce the carbonyls in the experiment as described previously (20). The absorbance of the spectrophotometric measurement of carbonyls was reduced to 16% with treatment, confirming the validity of the method.

Immunohistochemical Staining for Protein Carbonyls in the Lungs

Protein carbonyls were identified immunohistochemically with paraffin-embedded lung sections using specific antiserum against DNPH prepared with rabbits as described previously (20). The sections were incubated with DNPH solution for 30 min and then with anti-DNPH antibody for 1 h at room temperature. The sections were incubated with biotinylated anti-rabbit IgG antibody (Vector, Burlingame, CA). Antibody binding was detected using the Elite ABC Kit (Vector) and 3,3-diaminobenzidine tetrahydrochloride as the chromogen according to the manufacturer's instructions. As a negative control, the sections were incubated in 2% HCl without DNPH for 30 min before incubation with the primary antibody or in a solution containing 2% bovine serum albumin and 2% normal goat serum instead of the polyclonal anti-DNPH antibody. All the sections were counterstained with hematoxylin.

Chronic Exposure to Cigarette Smoke

Mice were exposed to cigarette smoke for 8 wk using the commercially marketed Peace nonfilter cigarettes (29 mg of tar and 2.5 mg of nicotine per cigarette; Japan Tobacco, Inc., Tokyo, Japan) and the Tobacco Smoke Inhalation Experiment System for small animals (Model SIS-CS; Shibata Scientific Technology, Tokyo, Japan) as described previously (14). We used 2.5% cigarette smoke diluted with compressed air (mass concentration of total particulate matter, 40.6 mg/m³). Three-month-old mice were exposed to diluted cigarette smoke (study group) or fresh air (control group) for 30 min/d, 5 d/wk, and for 8 wk.

Determination of Malondialdehyde in the Lungs

The measurement of malondialdehyde (MDA) in the supernatant of lung homogenates was performed using the Lipid Peroxidation Assay Kit (Calbiochem, San Diego, CA) according to the manufacturer's instructions.

Determination of Glutathione in the BALF

Total glutathione and oxidized glutathione contents in the BALF supernatants were measured using the Total Glutathione Quantification Kit (Dojindo Molecular Technologies, Kumamoto, Japan) according to the manufacturer's instructions.

Evaluation of Apoptosis with Immunohistochemistry for Anti-Single-stranded DNA and Anti-Activated Caspase-3 Antibodies

Apoptosis of lung cells was examined with immunohistochemistry using a rabbit polyclonal antibody against the single-stranded DNA (ssDNA;

Dako Cytomation, Carpinteria, CA) and anti-activated caspase-3 (Cell Signaling, Beverly, MA) as described previously (14).

Protein Assays

Total protein concentration was measured using the BCA Protein Assay Kit (Pierce, Rockford, IL) according to the manufacturer's instructions.

Statistical Analysis

The statistical significance of the data was determined using analysis of variance followed by Tukey's multiple comparison test. When applicable, the Mann-Whitney test was used. A *p* value of less than 0.05 was considered significant.

RESULTS

Age-related Changes in Body Weight, Morphometry of the Lungs, and Protein Carbonyls

Body weights increased with age in the SMP30Y/+ and SMP30Y/– mice, and there were no significant changes between both strains up to 6 mo of age. At 12 mo of age, the body weight of the SMP30Y/– mice were significantly less than that of the SMP30Y/+ mice (Table 1). Compared with the SMP30Y/+ mice, the SMP30Y/– mice had significantly greater MLI from 3 to 12 mo of age. There was a significant increase of MLI with aging in the lungs of the SMP30Y/– mice. On the other hand, the DI scores in both groups at every age were less than 10%, and no significant differences between the groups were recorded (Table 1). There were no inflammatory findings in the alveoli in both groups on histologic examination (data not shown).

We assessed protein oxidation in the lungs of SMP30Y/+ and SMP30Y/– mice by measuring the protein carbonyls, which are known as sensitive biomarkers of oxidative stress (21). In the lungs of SMP30Y/– mice, protein carbonyls were significantly increased in comparison with those of age-matched SMP30Y/+ mice. In addition, protein carbonyls tended to increase with age and significantly increased at 12 mo of age as compared with 1 mo of age (Figure 1). We performed immunohistochemistry with anti-DNPH antibody to detect the localization of protein carbonyls in the lungs and demonstrated that the majority of the protein carbonyls was evenly distributed and diffused in both strains but was apparently stronger in the SMP30Y/– mice (Figure 2).

Effect of Cigarette Smoke Exposure on the Morphometry of the Lungs

The body weights did not change significantly between the SMP30Y/+ and SMP30Y/– mice before and after exposure to

TABLE 1. AGE-RELATED CHANGES OF BODY WEIGHT AND LUNG MORPHOMETRY IN SMP30Y/+ AND SMP30Y/– MICE

	1 mo	3 mo	6 mo	12 mo
BW, g				
SMP30Y/+	22.1 ± 0.6	29.2 ± 0.9	33.3 ± 2.1	41.1 ± 2.3
SMP30Y/–	22.0 ± 0.6	28.0 ± 0.7	32.1 ± 3.2	37.6 ± 1.0*
MLI, μm				
SMP30Y/+	57.4 ± 4.4	57.7 ± 3.3	60.7 ± 1.6	61.5 ± 3.9
SMP30Y/–	58.6 ± 2.9	62.0 ± 1.4*†	67.1 ± 2.3*‡	67.6 ± 3.9*‡
DI, %				
SMP30Y/+	3.8 ± 0.7	4.2 ± 0.5	4.1 ± 1.1	4.5 ± 0.8
SMP30Y/–	4.2 ± 0.8	4.3 ± 0.5	4.5 ± 0.9	4.9 ± 1.0

Definition of abbreviations: BW = body weight; DI = destructive index; MLI = mean linear intercepts.

Data are presented as mean ± SD (*n* = 5 for each group).

* *p* < 0.05 versus age-matched SMP30Y/+ mice.

† *p* < 0.05 versus SMP30Y/– mice at 1 mo of age.

‡ *p* < 0.001 versus SMP30Y/– mice at 1 mo of age.

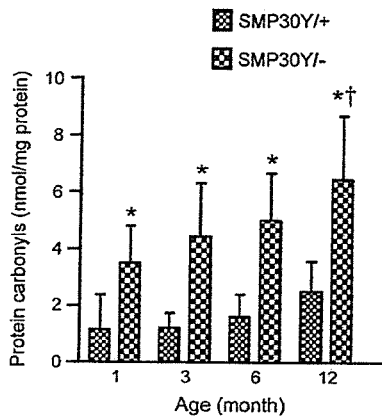


Figure 1. Age-related changes of protein carbonyls in the lungs of SMP30Y/+ and SMP30Y/- mice. In the lungs of the SMP30Y/- mice, protein carbonyls tended to increase with age ($p < 0.05$, compared with 1 mo of age) and were significantly greater than those of the age-matched SMP30Y/+ mice ($*p < 0.05$). Values are presented as mean \pm SD ($n = 5$ for each group).

cigarette smoke for 8 wk. In addition, exposure to cigarette smoke had no influence on body weight gain in both strains as compared with the air-exposed groups (data not shown).

Chronic exposure to cigarette smoke increased total cell counts in the BALF of both groups. No significant differences were observed in cell populations between air- and smoke-

exposed mice of both strains. The total protein concentration in the BALF increased significantly in the SMP30Y/- mice after chronic exposure to cigarette smoke but did not increase in the SMP30Y/+ mice. The smoke-exposed SMP30Y/- mice demonstrated significantly higher levels of protein concentration in BALF than the smoke-exposed SMP30Y/+ mice (Table 2).

Chronic exposure to cigarette smoke for 8 wk generated pulmonary emphysema in the SMP30Y/- mice but not in SMP30Y/+ mice. Histologic specimens of the lung tissues of smoke-exposed SMP30Y/- mice revealed marked airspace enlargement (i.e., an increase of MLI) accompanied with disruption of alveolar wall (i.e., an increase of DI; Figures 3 and 4). Morphometric examination of the lung specimen revealed that MLI was significantly greater in the smoke-exposed SMP30Y/- mice than in the air-exposed SMP30Y/- and SMP30Y/+ mice (Figure 4A): MLI increased to 23.3% in the SMP30Y/- mice after exposure to cigarette smoke and 5.4% for the SMP30Y/+ mice. Moreover, DI increased to more than 10%, a cut-off value indicating the occurrence of significant alveolar wall destruction (17), in the lungs from smoke-exposed SMP30Y/- mice, whereas no significant increase was observed in the SMP30Y/+ mice (Figure 4B).

Effect of Cigarette Smoke Exposure on Oxidative Stress in the Lungs

To investigate the effect of chronic cigarette smoke exposure on oxidative injury in the lungs, we measured protein carbonyls in the lungs of SMP30Y/+ and SMP30Y/- mice after exposure to air or cigarette smoke (Figure 5). Protein carbonyls tended to increase in the lungs of both strains after cigarette smoke exposure, although no significant statistical difference was noted ($p = 0.08$ and 0.07 between air and smoke exposure in SMP30Y/+ and SMP30Y/- mice, respectively). Protein carbonyls were significantly increased in the lungs of smoke-exposed SMP30Y/- mice than those of the smoke-exposed SMP30Y/+ mice. We also performed immunohistochemistry with anti-DNPH antibody on lung specimens from both strains after chronic exposure to cigarette smoke. Anti-DNPH antibody demonstrated an even and diffuse distribution of protein carbonyls in the lungs of both strains after exposure to cigarette smoke (data not shown), indicating that chronic smoke exposure seems to increase oxidative stress evenly throughout the lungs.

We assessed lipid peroxidation in the lungs by measuring MDA (Figure 6), which is known as one of the end products derived from peroxidation of polyunsaturated fatty acids and related esters (22). Baseline levels of MDA in the lung tissues from both strains (air-exposed) were almost identical. However, MDA significantly increased in the lungs of the SMP30Y/- mice after chronic cigarette smoke exposure, whereas no increase was demonstrated in the lungs of the SMP30Y/+ mice. We did not use the conventional thiobarbituric acid method to measure the MDA because of low specificity of this method. Instead, we used a method that allows us to assay MDA selectively by reacting with *N*-methyl-2-phenylindole in HCl, eliminating the potential inclusion of 4-hydroxylalkenals and alkanals in the measurement. The reaction produces a chromophore with maximum absorption at 586 nm, whereas the reaction with alkanals forms a product with maximum absorption at 505 nm (23).

We measured the content of total glutathione in BALF (Figure 7) because it is widely recognized as a major antioxidant in the lungs (24, 25). In SMP30Y/- mice, baseline level of total glutathione in BALF tended to be greater than that of the SMP30Y/+ mice, but no statistical significance was detected ($p = 0.06$). Chronic cigarette smoke exposure markedly up-regulated the amount of total glutathione in BALF from the SMP30Y/- mice ($p < 0.05$), whereas no effect of smoke exposure on the glutathione level was revealed in the SMP30Y/+ mice

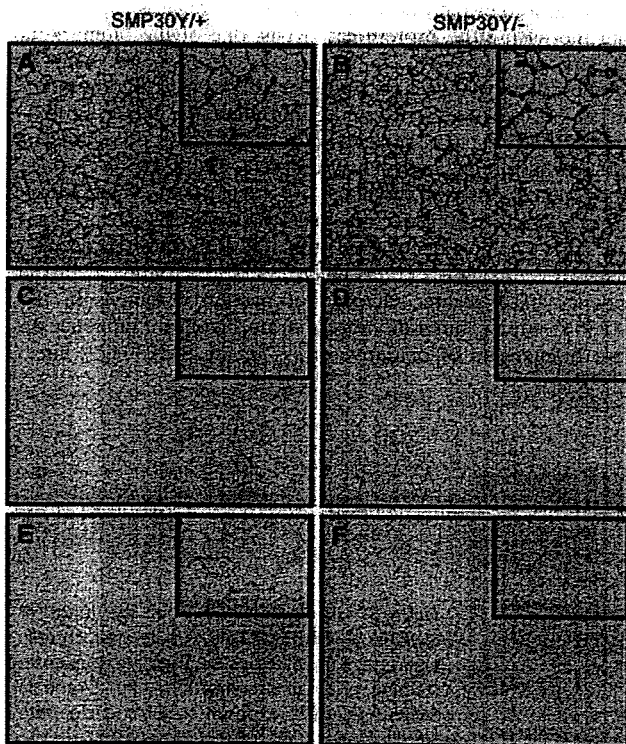


Figure 2. Immunohistochemistry for protein carbonyls in the lungs of SMP30Y/+ (A, C, and E) and SMP30Y/- (B, D, and F) mice (original magnification: $\times 100$). Insets are magnified views of the alveolar region ($\times 400$). Anti-2,4-dinitrophenylhydrazine (-DNPH) antibody analysis revealed that protein carbonyls were largely evenly and diffusely distributed in the lung tissues of both strains but apparently were stronger in the SMP30Y/- mice (A and B). As a control for immunostaining of the lung tissues, avoiding the derivatization process (C and D) and removing primary anti-DNPH antibody (E and F) resulted in the elimination of positive immunostaining.

TABLE 2. EFFECT OF CHRONIC CIGARETTE SMOKE EXPOSURE ON BRONCHOALVEOLAR LAVAGE FLUID ANALYSES IN SMP30Y/+ AND SMP30Y/- MICE

	SMP30Y/+		SMP30Y/-	
	Air Exposure	Smoke Exposure	Air Exposure	Smoke Exposure
Total cell count ($\times 10^5$)	0.5 \pm 0.2	1.5 \pm 0.3*	0.9 \pm 0.5	1.9 \pm 0.7 ^{††}
Cell differentiation				
Macrophages, %	90.3 \pm 7.3	88.8 \pm 4.0	87.0 \pm 10.2	84.0 \pm 9.7
Neutrophils, %	1.0 \pm 1.1	1.0 \pm 1.1	0.7 \pm 1.0	1.2 \pm 1.3
Lymphocytes, %	8.5 \pm 6.4	9.7 \pm 3.7	12.0 \pm 9.2	14.2 \pm 8.4
Total protein, μ g/ml	128.4 \pm 106.8	93.0 \pm 37.2	145.0 \pm 84.3	282.9 \pm 63.1 ^{†††}

Data are presented as mean \pm SD (n = 6 for each group).

* p < 0.001 versus SMP30Y/+ mice exposed to air.

[†] p < 0.01 versus SMP30Y/+ mice exposed to air.

^{††} p < 0.05 versus SMP30Y/- mice exposed to air.

^{†††} p < 0.05 versus SMP30Y/+ mice exposed to air.

^{††††} p < 0.001 versus SMP30Y/+ mice exposed to cigarette smoke.

(p = 0.79). We were able to detect oxidized glutathione in BALF from the cigarette smoke-exposed SMP30Y/- mice group but not from the other groups (i.e., less than the detection limit). The amount of oxidized glutathione detected in smoke-exposed SMP30Y/- mice was 7.91 nmol/mg protein (7.95% of total glutathione content).

Effect of Cigarette Smoke Exposure on Apoptosis of Lung Cells

Apoptosis of lung cells was analyzed to investigate the mechanisms of cigarette smoke-induced emphysema in the SMP30Y/- mice. Immunohistochemical examination using anti-single-stranded DNA antibody revealed that apoptosis was widely detected in bronchial and bronchiolar epithelial cells and alveolar septal cells and was significantly increased in all areas of the lungs of the cigarette smoke-exposed SMP30Y/- mice compared with the other groups (Figures 8A and 8B). On the other hand, there was no significant difference in the ratio of apoptotic nuclei detected between the air- and smoke-exposed SMP30Y/+ mice (p = 0.09) and between the air-exposed SMP30Y/+ and SMP30Y/- mice (p = 0.07). Chronic cigarette smoke-induced increase in apoptosis was further confirmed with immunohisto-

chemical analysis of caspase-3: Activated caspase-3 was detected in the lungs of cigarette smoke-exposed SMP30Y/- mice (Figure 8C) but not in the other groups (data not shown).

DISCUSSION

We have demonstrated that the oxidative stress in the lungs is greater in the SMP30Y/- mice than the SMP30Y/+ mice and tends to gradually increase in the SMP30Y/- mice as determined from measuring the protein carbonyls, one of the biomarkers for oxidative stress (26, 27), in the lungs. We did not measure the age-related changes of lipid peroxidation as another biomarker for oxidative stress and glutathione as a major antioxidant in BALF. At 5 mo of age, however, there was no significant statistical difference between SMP30Y/+ and SMP30Y/- mice in lipid peroxidation of the lungs (Figure 6; p = 0.76) and glutathione in BALF (Figure 7; p = 0.06). In contrast, chronic cigarette smoke exposure for 8 wk resulted in a marked increase of lipid peroxidation and up-regulation of glutathione, with the tendency of a greater increase of protein carbonyls in the SMP30Y/- mice, but it did not generate significant influence on these biomarkers of oxidative stress in the SMP30Y/+ mice.

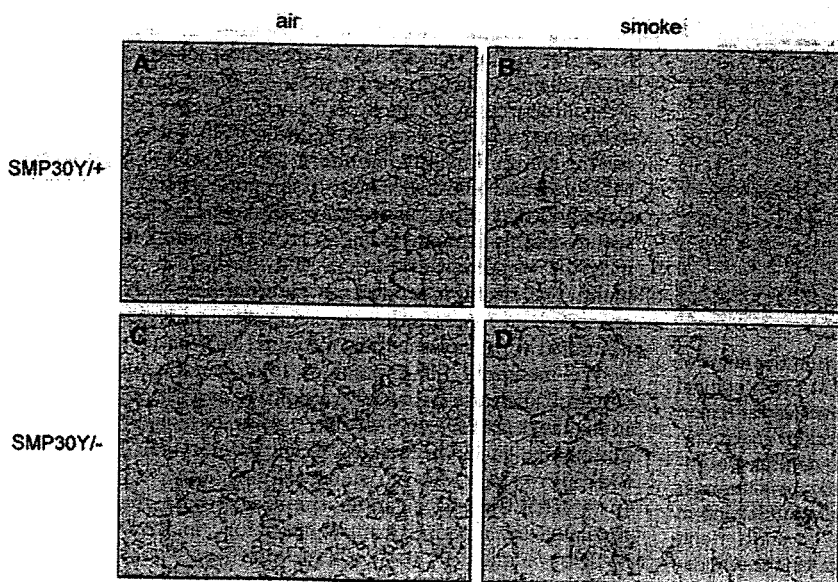


Figure 3. Histologic findings of the lungs of SMP30Y/+ and SMP30Y/- mice after chronic cigarette smoke exposure (hematoxylin-eosin staining; original magnification, $\times 50$). (A and B) SMP30Y/+ mice exposed to air and cigarette smoke, respectively. (C and D) SMP30Y/- mice, exposed to air and cigarette smoke, respectively. The lungs of the SMP30Y/- mice exposed to cigarette smoke revealed marked airspace enlargement and alveolar wall destruction (D).

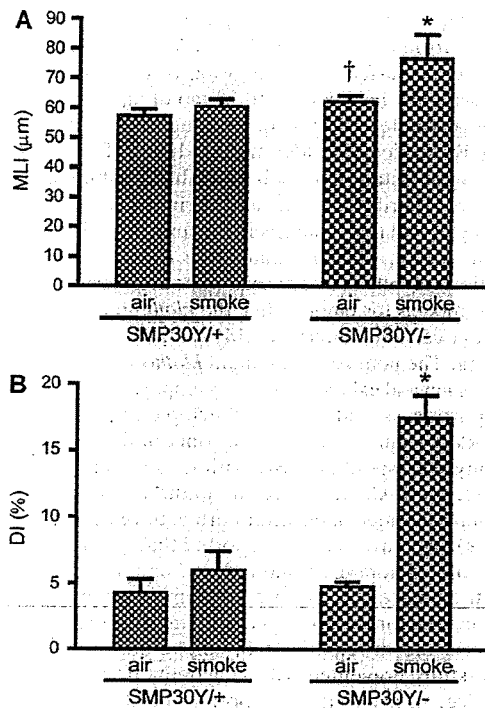


Figure 4. Morphometric findings of the lungs of SMP30Y+/+ and SMP30Y-/- mice after chronic cigarette smoke exposure. (A) Mean linear intercepts (MLI). Values are presented as mean \pm SD ($n = 6$ for each group). In the lungs of SMP30Y-/- mice exposed to cigarette smoke, MLI was significantly greater than the other groups ($*p < 0.05$). The MLI increased to 23.3% in the SMP30Y-/- mice and to 5.4% for the SMP30Y+/+ mice. The MLI in the SMP30Y-/- mice exposed to air was significantly greater than that of the SMP30Y+/+ mice exposed to air ($†p < 0.05$). (B) The destructive index (DI). Values are presented as mean \pm SD ($n = 6$ for each group). In the lungs of the SMP30Y-/- mice exposed to cigarette smoke, DI was increased more than 10% ($*p < 0.05$ compared with the other groups).

Our results indicate that the lack of a SMP30 molecule brings forth an endogenous mild oxidative stress situation in the lungs and makes the lungs highly susceptible to oxidative stress induced by smoke exposure even if exogenous oxidative stress is so mild that it does not generate oxidative proteins and lipids and up-regulate glutathione in BALF of the SMP30Y+/+ mice. In this context, SMP30Y-/- mice seem to be an excellent tool for investigating the pathophysiology of the lung associated with oxidant/antioxidant imbalance like smoke-induced emphysema and aging-related conditions like the senile lung.

The precise function of SMP30 in terms of oxidant and antioxidant balance remains undetermined. We demonstrated previously that SMP30 is localized in the nuclei in addition to the cytoplasm of cultured mouse hepatocytes and is similar in its amino acid sequence to bacterial and yeast RNA polymerases (28). Accordingly, SMP30 may regulate gene expression, and the lack of SMP30 may cause down-regulation of antioxidant enzymes. It has recently been reported that with gene targeting of the Nrf2 (nuclear factor, erythroid-derived 2, like 2), a transcription factor regulating gene expression involved in antioxidant defense, inflammation, and cellular apoptosis, plays an important role in the development of smoke-induced emphysema (29): Nrf2^{-/-} mice were demonstrated to be extremely susceptible to cigarette smoke-induced emphysema after 6 mo of expo-

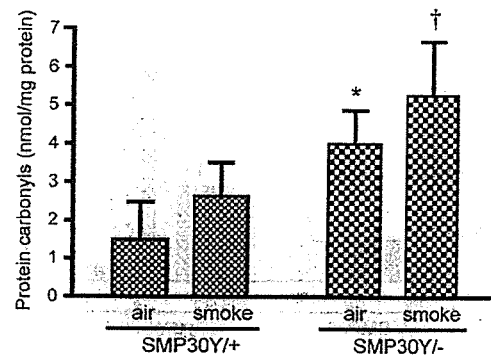


Figure 5. Protein carbonyls in the lungs of SMP30Y+/+ and SMP30Y-/- mice after chronic cigarette smoke exposure. Protein carbonyls in the lungs of SMP30Y-/- mice were significantly greater in the air- and smoke-exposed groups than those of SMP30Y+/+ mice ($*p < 0.05$ compared with SMP30Y+/+ mice air-exposed group, and $†p < 0.05$ compared with SMP30Y+/+ mice smoke-exposed group). Values are presented as mean \pm SD ($n = 6$ for each group).

sure. Glutathione is a potent antioxidant in the lungs and is highly concentrated in the epithelial lining fluid (24, 25). The transcription of the gene for α -glutamylcysteine synthetase, the rate-limiting enzyme for glutathione synthesis, is markedly up-regulated by chronic cigarette smoke exposure (30). Cigarette smoke-induced up-regulation of γ -glutamylcysteine synthetase has been reported to be mediated by the redox-sensitive transcription factor, activating protein-1 (31, 32). Although we did not directly measure enzyme activities, SMP30 seems not to be involved in the regulation of γ -glutamylcysteine synthetase because the glutathione content in BALF was markedly increased after 8 wk of smoke exposure in the SMP30Y-/- mice. Moreover, the activity of glutathione reductase is not likely to be affected in the SMP30Y-/- mice because the fraction of the oxidized form of glutathione is approximately 8% of the increased total glutathione content of the BALF. Because γ -glutamylcysteine synthetase and glutathione reductase are involved in the Nrf2 pathway (29), SMP30 may not have functional cross-talk with Nrf2. On the other hand, we previously reported lipid deposition and degeneration of mitochondria in the liver, kidney, and

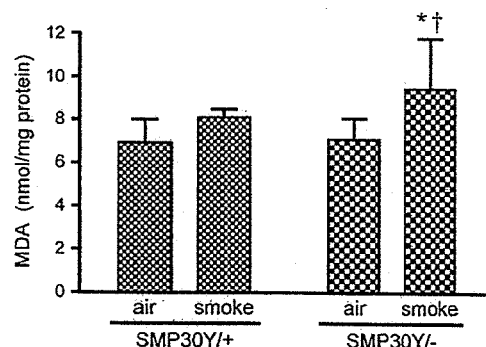


Figure 6. Malondialdehyde (MDA) in the lungs of SMP30Y+/+ and SMP30Y-/- mice after chronic cigarette smoke exposure. MDA was significantly increased in the lungs of SMP30Y-/- mice exposed to cigarette smoke as compared with those of the air-exposed SMP30Y+/+ ($*p < 0.05$) and SMP30Y-/- ($†p < 0.05$) mice. Values are presented as mean \pm SD ($n = 6$ for each group).

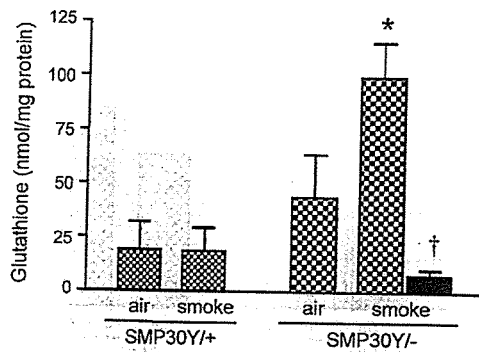


Figure 7. Total glutathione content in a bronchoalveolar lavage fluid (BALF) specimen of SMP30Y^{+/+} and SMP30Y^{-/-} mice after chronic cigarette smoke exposure. Total glutathione content was significantly up-regulated in the SMP30Y^{-/-} mice exposed to cigarette smoke as compared with the other groups (* $p < 0.05$). Values are presented as mean \pm SD ($n = 6$ for each group). †Oxidized glutathione was detected only in BALF from the smoke-exposed SMP30Y^{-/-} mice but not from the other groups (i.e., less than the detection limit). The amount of oxidized glutathione detected in the smoke-exposed SMP30Y^{-/-} mice was 7.91 nmol/mg protein (7.95% of total glutathione content).

submandibular gland of SMP30Y^{-/-} mice (8, 33, 34). The mitochondrion is speculated to be the main source of endogenous intracellular oxidant through a leak of an electron from the mitochondrial respiratory chain, but it has antioxidant enzymes (Mn-superoxide dismutase and glutathione peroxidase) that can attenuate oxidative stress. Recent studies have highlighted the important role of mitochondrial proteolytic enzymes in providing resistance to oxidative stress (35). Accordingly, the mitochondrial degeneration observed in the SMP30Y^{-/-} mice may be involved in the susceptibility of the lungs to oxidative stress. However, our preliminary electron microscopic analysis of the lungs did not detect structural abnormalities in the SMP30Y^{-/-} mice as compared with lungs of the SMP30Y^{+/+} mice (data not shown). Further studies are needed to elucidate the precise role of SMP30 in oxidant/antioxidant balance by examining the gene expression profiles of the lungs from the SMP30Y^{-/-} mice with special reference to mitochondrial antioxidant enzymes and proteolytic enzymes.

We previously reported that SMP30Y^{-/-} is a novel murine model of senile lung because senile lungs develop spontaneous airspace enlargement without parenchymal destruction (5). This was confirmed in this study because SMP30Y^{-/-} mice showed significantly greater MLI at 3 mo of age than SMP30Y^{+/+} mice. The SMP30Y^{-/-} mouse seems to be not only a murine model of senile lung but also a murine model of cigarette smoke-induced emphysema. SMP30Y^{-/-} mice are markedly susceptible to cigarette smoke, and smoke exposure for 8 wk was sufficient to develop cigarette smoke-induced pulmonary emphysema with marked airspace enlargement and parenchymal destruction. Although some animal models have been reported to develop cigarette smoke-induced pulmonary emphysema, most animal models required a longer period of cigarette smoke exposure, generally 6 or 7 mo, to generate smoke-induced emphysema. Because cigarette smoke-induced pulmonary emphysema in humans usually occurs in the elderly population, we considered that aging of the lung can be an important factor and should be incorporated into the experimental animal model for such condition. The effect of age on lung morphometry (36) and in the development of chronic cigarette smoke-induced lung pathology (37) has been reported. In BALB/cN^{ia} mice, alveo-

lar multiplication seemed to be completed by 38 d of age; interalveolar pore formation increased until 9 mo of age; and lung volume, alveolar surface area, and total volume of alveolar wall increased with age between 9 and 28 mo of age, which is postulated to be attributed to aging of the lungs (36). It was reported that in C57BL/6 mice, the older mice (8–10 mo of age) developed pathologic manifestations closely resembling pulmonary fibrosis and developed peribronchiolar and perivascular accumulations of lymphocytes and macrophages in the lungs after 9 mo exposure to cigarette smoke, whereas young mice (2 mo of age) revealed accumulations of inflammatory cells without fibrosis (37). Among other mice models for emphysema, the *klotho* mutant mouse and senescence-accelerated mouse (SAM) are unique due to their biological aging. The homozygous mutant *klotho* mice demonstrate a shorter life span and exhibit pulmonary emphysema, arteriosclerosis, osteoporosis, skin atrophy, and ectopic calcifications. However, the *klotho* mutant mice are distinct in developing pulmonary emphysema spontaneously without smoking (38). On the other hand, the SAM mice are the naturally occurring animal models for accelerated aging after normal development and maturation (39). We have recently reported that the SAMP1 mouse is capable of developing smoke-induced emphysema after 8 wk of cigarette smoke exposure. We also demonstrated that SAMP1 mice can be used for experiments involving therapeutic intervention because the development of smoke-induced emphysema was successfully prevented with concomitant administration of tomato juice, which contains a potent nutritional antioxidant, lycopene (14). In this context, SMP30Y^{-/-} mice further illustrate the significance of biological aging of the lungs in the development of cigarette smoke-induced pulmonary emphysema and may be considered as valuable animal models for smoke-induced emphysema.

Several mechanisms are likely to be involved in the development of cigarette smoke-induced pulmonary emphysema. An increase of oxidative stress to the lungs may be associated with many of the pathogenic processes, such as direct injury to lung cells, mucus hypersecretion, inactivation of antiproteases, enhancing lung inflammation through activation of redox-sensitive transcription factors, and apoptosis of lung cells (11). In the present study, chronic smoke exposure increased total cell count in SMP30Y^{+/+} and SMP30Y^{-/-} mice. On the other hand, SMP30Y^{-/-} mice showed total protein level in BALF increased twofold from the baseline level after smoke exposure, whereas no change was detected in SMP30Y^{+/+} mice. These findings may suggest that the inflammation in the lungs induced by chronic smoke exposure may be more pronounced in SMP30Y^{-/-} mice than in SMP30Y^{+/+} mice, although we did not measure any other parameters of inflammation. Pulmonary emphysema can be generated without apparent inflammation, and it has recently been recognized that alveolar cell apoptosis could be one of the crucial process in emphysema: Direct instillation of activated caspase-3 (40) or vascular endothelial cell apoptosis resulting from the blockade of the vascular endothelial growth factor receptors (41) has been demonstrated to result in emphysema. As we reported previously that hepatocytes from SMP30Y^{-/-} mice are susceptible to apoptosis (7), we confirmed in this study that lungs cells are also susceptible to apoptosis triggered by oxidative stress. Accordingly, SMP30Y^{-/-} mice may be ideal animal models for cigarette smoke-induced emphysema in terms of investigating mutual interactions among apoptosis, oxidative stress, and inflammation, which is proposed as the mechanism for irreversible progression of parenchymal destruction (42, 43). Recently, up-regulation of lung ceramide, a second messenger lipid, has been reported to be a key pathogenic element in these mutual interactions (44). Because we have previously demonstrated that abnormal lipid metabolism occurs in the liver of the SMP30Y^{-/-} mice

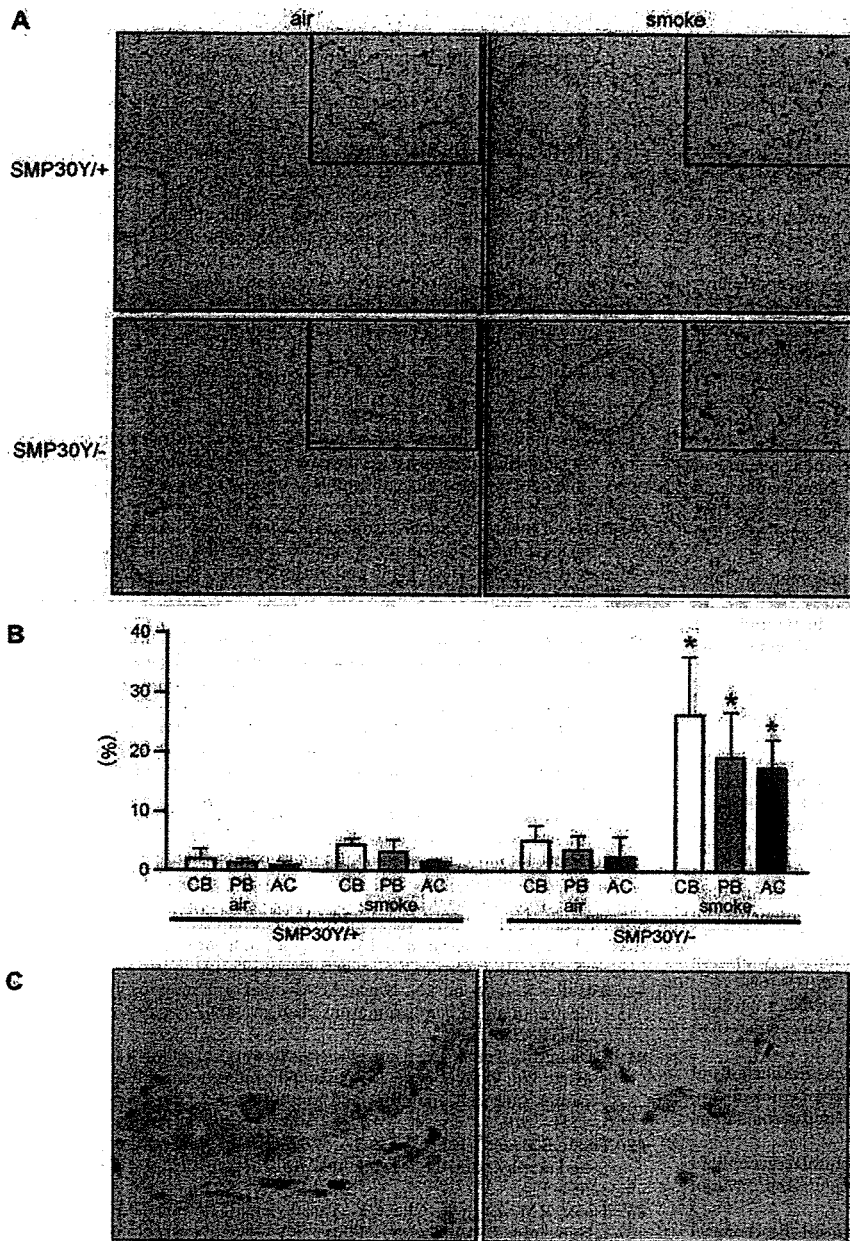


Figure 8. Immunohistochemical detection of apoptosis in the lungs of SMP30Y^{+/+} and SMP30Y^{-/-} mice after cigarette smoke exposure. (A) Representative results of immunohistochemistry for single-stranded DNA (original magnification: ×50). Insets are magnified views of the alveolar region (×200). Note that positive immunostaining (nucleus stained brown) for DNA strand breaks was revealed in airway epithelial and alveolar wall cells in the lungs of SMP30Y^{-/-} mice after cigarette smoke exposure compared with the other groups. (B) Immunoreactive nuclei for anti-single-stranded DNA antibody were counted in three areas and expressed as the positive ratio (%) of total nuclei counted. AC = alveolar septal cells; CB = bronchial cells in the central airway; PB = bronchiolar cells adjacent to alveolar duct. The ratio of positively immunostained nuclei in all areas of the lungs of cigarette smoke-exposed SMP30Y^{-/-} mice was significantly higher than those of the other groups (*p < 0.001). Values are presented as mean ± SD (n = 6 for each group). (C) Representative results of immunohistochemistry for the activated caspase-3 in the lungs of the SMP30Y^{-/-} mice after cigarette smoke exposure (left: bronchial cells; right: alveolar septal cells; original magnification ×200). Positive reactions were identified in the lungs of cigarette smoke-exposed SMP30Y^{-/-} mice but were rarely seen in the lungs of the other groups.

(8), we need to examine whether SMP30 may be involved in the regulation of lung ceramide as the next step of our study.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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References

- Fujita T, Uchida K, Maruyama N. Purification of senescence marker protein-30 (SMP30) and its androgen-independent decrease with age in the rat liver. *Biochim Biophys Acta* 1992;1116:122-128.
- Fujita T, Shirasawa T, Uchida K, Maruyama N. Isolation of cDNA clone encoding rat senescence marker protein-30 (SMP30) and its tissue distribution. *Biochim Biophys Acta* 1992;1132:297-305.
- Fujita T, Shirasawa T, Uchida K, Maruyama N. Gene regulation of senescence marker protein-30 (SMP30): coordinated up-regulation

with tissue maturation and gradual down-regulation with aging. *Mech Ageing Dev* 1996;87:219-229.

- Fujita T, Shirasawa T, Maruyama N. Isolation and characterization of genomic and cDNA clones encoding mouse senescence marker protein-30 (SMP30). *Biochim Biophys Acta* 1996;1308:49-57.
- Mori T, Ishigami A, Scyama K, Onai R, Kubo S, Shimizu K, Maruyama N, Fukuchi Y. Senescence marker protein-30 knockout mouse as a novel murine model of senile lung. *Pathol Int* 2004;54:167-173.
- Fujita T, Mandel JL, Shirasawa T, Hino O, Shirai T, Maruyama N. Isolation of cDNA clone encoding human homologue of senescence marker protein-30 (SMP30) and its location on the X chromosome. *Biochim Biophys Acta* 1995;1263:249-252.
- Ishigami A, Fujita T, Handa S, Shirasawa T, Koseki H, Kitamura T, Enomoto N, Sato N, Shimosawa T, Maruyama N. Senescence marker protein-30 knockout mouse liver is highly susceptible to tumor necrosis factor-alpha- and Fas-mediated apoptosis. *Am J Pathol* 2002;161:1273-1281.
- Ishigami A, Kondo Y, Namba R, Ohsawa T, Handa S, Kubo S, Akita M, Maruyama N. SMP30 deficiency in mice causes an accumulation of

- neutral lipids and phospholipids in the liver and shortens the life span. *Biochem Biophys Res Commun* 2004;315:575-580.
9. Fujita T, Inoue H, Kitamura T, Sato N, Shimosawa T, Maruyama N. Senescence marker protein-30 (SMP30) rescues cell death by enhancing plasma membrane Ca(2+)-pumping activity in Hep G2 cells. *Biochem Biophys Res Commun* 1998;250:374-380.
 10. Junqueira VB, Barros SB, Chan SS, Rodrigues L, Giavarotti L, Abud RL, Deucher GP. Aging and oxidative stress. *Mol Aspects Med* 2004; 25:5-16.
 11. MacNee W. Pulmonary and systemic oxidant/antioxidant imbalance in chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2005;2: 50-60.
 12. Rahman I, van Schadewijk AA, Crowther AJ, Hiemstra PS, Stolk J, MacNee W, De Boer WI. 4-Hydroxy-2-nonenal, a specific lipid peroxidation product, is elevated in lungs of patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2002;166:490-495.
 13. Barreiro E, de la Puente B, Minguella J, Corominas JM, Serrano S, Hussain SN, Gea J. Oxidative stress and respiratory muscle dysfunction in severe chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2005;171:1116-1124.
 14. Kasagi S, Seyama K, Mori H, Souma S, Sato T, Akiyoshi T, Suganuma H, Fukuchi Y. Tomato juice prevents from developing emphysema induced by chronic exposure to tobacco smoke in senescence-accelerated mouse P1 strain. *Am J Physiol Lung Cell Mol Physiol* 2006;290: 396-404.
 15. Thurlbeck WM. The internal surface area of nonemphysematous lungs. *Am Rev Respir Dis* 1967;95:765-773.
 16. Eidelman DH, Bellofiore S, Chiche D, Cosio MG, Martin JG. Behavior of morphometric indices in pancreatic elastase-induced emphysema in rats. *Lung* 1990;168:159-169.
 17. Teramoto S, Fukuchi Y, Uejima Y, Teramoto K, Oka T, Orimo H. A novel model of senile lung: senescence-accelerated mouse (SAM). *Am J Respir Crit Care Med* 1994;150:238-244.
 18. Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, Ahn BW, Shaltiel S, Stadtman ER. Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol* 1990;186:464-478.
 19. Levine RL, Williams JA, Stadtman ER, Shacter E. Carbonyl assays for determination of oxidatively modified proteins. *Methods Enzymol* 1994;233:346-357.
 20. Nakamura A, Goto S. Analysis of protein carbonyls with 2,4-dinitrophenyl hydrazine and its antibodies by immunoblot in two-dimensional gel electrophoresis. *J Biochem (Tokyo)* 1996;119:768-774.
 21. Dalle-Donne I, Giustarini D, Colombo R, Rossi R, Milzani A. Protein carbonylation in human diseases. *Trends Mol Med* 2003;9:169-176.
 22. Liu J, Yeo HC, Doniger SJ, Ames BN. Assay of aldehydes from lipid peroxidation: gas chromatography-mass spectrometry compared to thiobarbituric acid. *Anal Biochem* 1997;245:161-166.
 23. Erdelmeier I, Gerard-Monnier D, Yadan JC, Chaudiere J. Reactions of N-methyl-2-phenylindole with malondialdehyde and 4-hydroxyalkenals: mechanistic aspects of the colorimetric assay of lipid peroxidation. *Chem Res Toxicol* 1998;11:1184-1194.
 24. Cantin AM, North SL, Hubbard RC, Crystal RG. Normal alveolar epithelial lining fluid contains high levels of glutathione. *J Appl Physiol* 1987;63:152-157.
 25. Teramoto S, Fukuchi Y, Uejima Y, Teramoto K, Orimo H. Biochemical characteristics of lungs in senescence-accelerated mouse (SAM). *Eur Respir J* 1995;8:450-456.
 26. Stadtman ER, Levine RL. Protein oxidation. *Ann N Y Acad Sci* 2000;899: 191-208.
 27. Goto S, Nakamura A. Age-associated, oxidatively modified proteins: a critical evaluation. *Age (Oman)* 1997;20:81-89.
 28. Ishigami A, Handa S, Maruyama N, Supakar PC. Nuclear localization of senescence marker protein-30, SMP30, in cultured mouse hepatocytes and its similarity to RNA polymerase. *Biosci Biotechnol Biochem* 2003;67:158-160.
 29. Rangasamy T, Cho CY, Thimmulappa RK, Zhen L, Srisuma SS, Kensler TW, Yamamoto M, Petrache I, Tudor RM, Biswal S. Genetic ablation of Nrf2 enhances susceptibility to cigarette smoke-induced emphysema in mice. *J Clin Invest* 2004;114:1248-1259.
 30. Rahman I, Smith CA, Lawson MF, Harrison DJ, MacNee W. Induction of gamma-glutamylcysteine synthetase by cigarette smoke is associated with AP-1 in human alveolar epithelial cells. *FEBS Lett* 1996;396: 21-25.
 31. Rahman I, Antonicelli F, MacNee W. Molecular mechanism of the regulation of glutathione synthesis by tumor necrosis factor-alpha and dexamethasone in human alveolar epithelial cells. *J Biol Chem* 1999; 274:5088-5096.
 32. Rahman I, Smith CA, Antonicelli F, MacNee W. Characterisation of gamma-glutamylcysteine synthetase-heavy subunit promoter: a critical role for AP-1. *FEBS Lett* 1998;427:129-133.
 33. Maruyama N, Ishigami A, Kuramoto M, Handa S, Kubo S, Imasawa T, Seyama K, Shimosawa T, Kasahara Y. Senescence marker protein-30 knockout mouse as an aging model. *Ann N Y Acad Sci* 2004;1019:383-387.
 34. Ishii K, Tsubaki T, Fujita K, Ishigami A, Maruyama N, Akita M. Immunohistochemical localization of senescence marker protein-30 (SMP30) in the submandibular gland and ultrastructural changes of the granular duct cells in SMP30 knockout mice. *Histol Histopathol* 2005;20:761-768.
 35. Bota DA, Davies KJ. Protein degradation in mitochondria: implications for oxidative stress, aging and disease: a novel etiological classification of mitochondrial proteolytic disorders. *Mitochondrion* 2001;1:33-49.
 36. Kawakami M, Paul JL, Thurlbeck WM. The effect of age on lung structure in male BALB/cNnJa inbred mice. *Am J Anat* 1984;170:1-21.
 37. Matulionis DH. Chronic cigarette smoke inhalation and aging in mice: I. Morphologic and functional lung abnormalities. *Exp Lung Res* 1984; 7:237-256.
 38. Suga T, Kurabayashi M, Sando Y, Ohyama Y, Maeno T, Maeno Y, Aizawa H, Matsumura Y, Kuwaki T, Kuro OM, et al. Disruption of the klotho gene causes pulmonary emphysema in mice: defect in maintenance of pulmonary integrity during postnatal life. *Am J Respir Cell Mol Biol* 2000;22:26-33.
 39. Takoda T, Hosokawa M, Higuchi K, Hosono M, Akiyuchi I, Katoh H. A novel murine model of aging, Senescence-Accelerated Mouse (SAM). *Arch Gerontol Geriatr* 1994;19:185-192.
 40. Aoshiba K, Yokohori N, Nagai A. Alveolar wall apoptosis causes lung destruction and emphysematous changes. *Am J Respir Cell Mol Biol* 2003;28:555-562.
 41. Kasahara Y, Tudor RM, Taraseviciene-Stewart L, Le Cras TD, Abman S, Hirth PK, Waltenerberger J, Voelkel NF. Inhibition of VEGF receptors causes lung cell apoptosis and emphysema. *J Clin Invest* 2000;106: 1311-1319.
 42. Tudor RM, Petrache I, Elias JA, Voelkel NF, Henson PM. Apoptosis and emphysema: the missing link. *Am J Respir Cell Mol Biol* 2003;28: 551-554.
 43. Tudor RM, Zhen L, Cho CY, Taraseviciene-Stewart L, Kasahara Y, Salvemini D, Voelkel NF, Flores SC. Oxidative stress and apoptosis interact and cause emphysema due to vascular endothelial growth factor receptor blockade. *Am J Respir Cell Mol Biol* 2003;29:88-97.
 44. Petrache I, Natarajan V, Zhen L, Medler TR, Richter AT, Cho C, Hubbard WC, Berdyshev EV, Tudor RM. Ceramide upregulation causes pulmonary cell apoptosis and emphysema-like disease in mice. *Nat Med* 2005;11:491-498.

Original Research Communication

The Redox-Sensitive DNA Binding Sites Responsible for Age-Related Downregulation of SMP30 by ERK Pathway and Reversal by Calorie Restriction

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BYUNG PAL YU,^{2,4} and HAE YOUNG CHUNG,^{1,2}

ABSTRACT

It was recently found that age-related changes in SMP30 expression can be modulated by antioxidative action. In the current study, the modulation of SMP30 gene expression was explored by (a) antioxidative calorie restriction (CR), (b) proinflammatory lipopolysaccharide (LPS), in aged rat, (c) oxidative stress promoter, *tert*-butylhydroperoxide (*t*-BHP)-injected mouse, and (d) *t*-BHP-treated Ac2F cells. Utilizing EMSA, particular attention was given to the binding activity of unidentified transcription factor in sites 3 and 5 that are located in -800 bp of the SMP30 promoter. Results showed that CR prevented the age-related decrease in SMP30 expression, and also showed that SMP30 gene expression and binding activities of sites 3 and 5 decreased with treatments of *t*-BHP or LPS. These findings were confirmed by the antioxidant NAC and ERK-specific inhibitor PD098059 that blunted decreased SMP30 gene expression and binding activity of sites 3 and 5 by *t*-BHP in Ac2F cell system. Our data strongly indicate that the SMP30 transcriptional process is redox-sensitive and its modulation occurs at DNA binding sites 3 and 5 in the promoter region. Perhaps a more significant finding of the present study is that the downregulation of SMP30 is likely involved in ERK signal pathway. *Antioxid. Redox Signal.* 8, 671-680.

INTRODUCTION

ACCUMULATED DATA strongly indicate that a major cause of deterioration of physiological function occurring during aging is due to upregulated oxidative stress. Most senescence-related changes in the expression of functional cellular proteins are considered to consist of multiple deteriorative factors influencing various cellular activities and homeostasis during aging (35).

The newly characterized senescence marker protein-30 (SMP30) is an important Ca²⁺-regulating protein present in hepatocytes and renal tubular epithelia that is known to decrease during the aging process. Its molecular size is about 30

kDa with a pI value of 4.9 (7, 15). Since its function includes maintaining Ca²⁺ homeostasis by enhancing plasma membrane Ca²⁺-pumping activity (13), the downregulation of SMP30 expression in aged tissue may induce the dysregulation of Ca²⁺ homeostasis, resulting in alterations of the signaling system (6) and cellular function (4, 22, 28). Moreover, the age-related downregulation of SMP30 has been reported to cogitate under elevated inflammatory conditions during aging (6, 9). Therefore, an age-associated decrease of SMP30 in the aged liver and kidney can be expected to predispose the organ to cell damage with age (19).

Experimental findings from studies using SMP30-deficient (SMP30^{-/-}) mice have established a causal rela-

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tion between the age-associated decrease in SMP30 and age-associated organ disorders (16). In the SMP30^{-/-} mouse, the liver proved to be more susceptible to the inflammatory cytokine TNF α - and Fas-mediated apoptosis than wild-type mice, both *in vitro* and *in vivo* (14). These investigators showed that SMP30 functions to protect cells from apoptosis and that reduced SMP30 expression accounts for age-associated deterioration of cellular functions that consequently enhances and aged organism's susceptibility to harmful stimuli such as inflammatory mediator, TNF α (14, 16).

Although SMP30 recently was suggested to exert an effective action on cell proliferation (12), the possible underlying cellular mechanisms were not well defined. At the molecular level, there are several binding sites of unidentified nuclear proteins in the SMP30 promoter region that participate in the differential expression of SMP30 in concert with other regulatory factors associated with aging (6). For example, the DNA binding activity of Sp1 declines during aging in rodent liver (1, 31). This may contribute to the downregulation of SMP30 gene expression in aged animals (6, 19). To better understand the molecular mechanism of the differential expression of SMP30 gene, Supakar *et al.* (32) investigated the SMP30 promoter region using DNase I foot-printing and electrophoretic mobility shift assay (EMSA) (32). These investigators identified several DNA binding sites of nuclear transcription factors that interact within 0.8 kb of the SMP30 gene promoter.

Calorie restriction (CR) has been shown to be one of most effective modulators of oxidative stress (35), which according to the evidence, shows lifespan prolongation that correlates with the differential expression of functional proteins involved in energy metabolism, stress responses, and Ca²⁺ regulation (23). In addition, the administration of free radical scavengers to an aged animal is shown to reverse protein oxidation levels and to restore the animal's redox state, which results in reduced age-related deterioration (8). Moreover, CR is reported to regulate the increase of oxidative stress-related cellular kinases, such as p38, ERK, and JNK, which are also calcium-related cell signaling kinases. While these kinases are shown to be upregulated by both oxidative stress and increased calcium levels, they are downregulated by CR (11, 20).

We report here in our experimental data on the age-related decrease of SMP30 with respect to the effects of age-related increases in oxidative stress and its antioxidative action. To characterize the effects of the redox status on SMP30 expression and its promoter activity, we investigate whether the transcriptional downregulation of SMP30 causes a decrease unidentified transcriptional binding activity at sites 3 and 5, which are suggested to be regulated by oxidative stress during aging. In addition, we attempt to show that the downregulation of SMP30 gene expression and the binding site activities are implicated in the ERK pathway.

MATERIALS AND METHODS

Materials

Reagents. Anti-SMP30 primary antibody was kindly supported by Dr. Maruyama at Japan's Tokyo Metropolitan In-

stitute of Gerontology. A horseradish peroxidase-conjugated donkey anti-rabbit antibody was obtained from Amersham (Amersham, Bucks, UK). Polyvinylidene difluoride (PVDF) membranes were obtained from Millipore Corporation (Bedford, MA, USA). The radionucleotide [γ -³²P]-ATP was obtained from Amersham (Bucks, UK). Chemicals, both *tert*-butylhydroperoxide (*t*-BHP) and lipopolysaccharide (LPS) were purchased from Sigma (St. Louis, MO, USA). Specific inhibitors for each of the kinases were obtained from Calbiochem (Darmstadt, Germany). All other chemicals were of the highest purity available from either Sigma Chemical Co. or Junsei Chemical Co. (Tokyo, Japan).

Animals. Specific pathogen-free male Fischer 344 rats were 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. All rats were fed *ad libitum* (AL) until 6 weeks of age, at which time they were divided into two groups: *ad libitum*-fed control group and calorie-restricted (CR) group. In the CR group, food intake was restricted to 60% of the food intake of the control group. Rats at 6, 12, 18, and 24 months of age were used in this study. For the inflammatory-induction experiments, rats at 13 and 31 months of age were intraperitoneally (i.p.) injected with 5 mg/kg LPS, 5 h before sacrifice.

In a separate experimentation to test the challenge of oxidative stress, male ICR mice were at 5 weeks of age were i.p. injected at two dose levels of *t*-BHP, 30 and 70 mg/kg, 5 h before sacrifice.

Methods

Tissue preparation. Livers from male, specific pathogen-free, Fischer 344 rats that were raised in the barrier facilities at the University of Texas Health Science Center at San Antonio, Department of Physiology, San Antonio, TX, USA, were used in the study. Complete descriptions of the housing, care, and feeding of the animals have been reported elsewhere (35). At 6, 12, 18, and 24 months of age, the rats were decapitated, their chests were opened, and their livers were quickly excised and immersed in ice-cold isotonic saline.

For the LPS challenge, rats at 13 and 31 months of age were i.p.-injected (5 mg/kg) with LPS. At 5 h following injection, the rats were decapitated and livers were quickly removed, and were immediately immersed in liquid nitrogen and stored at -80°C. The procedures used for the *t*-BHP injected mice were consistent with those used for sacrifice and tissue removal from rats.

To obtain a cytosol fraction, tissues of rats and mice were homogenized in ice-cold homogenization solution (50 mM potassium phosphate buffer containing 1 mM EDTA, 1 mM *p*-aminobenzamide, 1 μ M pepstatin, pH 7.4) and centrifuged at 900 g at 4°C for 15 min. Then, the supernatant was additionally centrifuged at 12,000 g at 4°C for 15 min.

Cell culture. A normal rat liver cell line, Ac2F was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in Dulbecco's modified Eagle's

medium (DMEM) medium (Nissui Co., Tokyo, Japan) supplemented with 10% heat-inactivated (56°C for 30 min) fetal bovine serum (HyClone, Logan, UT, USA), 5.84 mg/ml glutamine, 0.25 µg/ml amphotericin, and 100 unit/ml penicillin-streptomycin solution (Gibco, Grand Island, NY, USA) and adjusted to pH 7.4–7.6 by NaHCO₃. Oxygen tensions in the incubator were 140 mmHg (5% CO₂, 20% O₂, 75% N₂; normoxia) in a humidified incubator at 37°C. Each experiment is used at 80% confluence of cell.

Nuclear extract preparation. *Extracts from rat tissues.* All solutions, tubes, and centrifuges were maintained at 0–4°C. For each nuclear extract preparation, three livers were used. The preparation of nuclear extracts was based on previous publications (9). The nuclear extract was frozen at –70°C in aliquots until electrophoretic mobility shift assay (EMSA) was done.

Extracts from cultured liver. Nuclear extracts were prepared and assays were performed by a modified procedures as previously described (17) using cultured liver cells (Ac2F). Cells were harvested, washed with phosphate-buffered saline, and subjected to centrifugation at 3000 rpm at 4°C for 5 min. The washed cells were resuspended in “hypotonic buffer” [10 mM Tris-HCl, pH 8.0, containing 1.5 mM MgCl₂, 1 mM DTT, 0.1% Nonidet-P40, 5 µg/ml pepstatin, 5 µg/ml aprotinin, and 10 µM N-CBZ-LEU-LEU-LEU-AL (CBZ-LLL)] and let stand on ice for 20 min. Then cells were subjected to centrifugation at 12,000 rpm at 4°C for 15 min. The supernatants were used as cytosol. The pellets were resuspended in “nuclear extract buffer” (10 mM Tris-HCl, pH 8.0, containing 50 mM KCl, 300 mM NaCl, 1 mM DTT, 5 µg/ml pepstatin, 5 µg/ml aprotinin, and 10 µM CBZ-LLL). After incubation on ice for 30 min, the sample was subjected to centrifugation at 12,000 rpm at 4°C for 30 min. The supernatant was collected directly and defined as the nuclear extract.

Western blot. To investigate changes of gene expression in SMP30, we used Western blot to examine the cytosol fractions of livers and cell lysates. Samples were boiled for 5 min with a gel loading buffer. Total protein equivalents for each sample were resolved on a 12% SDS polyacrylamide mini gel, which were transferred to PVDF membrane at 100 voltage for 1.5 h. The membrane was immediately placed in blocking buffer (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 for 1–2 h at 25°C, followed by another incubation with a secondary antibody, a horseradish peroxidase-conjugated donkey anti-rabbit antibody (1:5000; Amersham). Antibody labeling was detected using enhanced chemiluminescence (ECL) per the manufacturer’s instructions and exposed to hyperfilm (Amersham).

Electrophoretic mobility shift assay (EMSA).

The electrophoretic mobility shift assay (EMSA) method was used to characterize the binding activities of known and novel transcription factors of the SMP30 promoter in nuclear extracts (21, 32). All of the oligonucleotides were synthesized from Bioneer (Daejeon, Korea); their sequences are shown in Table 1 (32). Complementary oligonucleotides synthesized separately were annealed in 20 mM Tris-HCl (pH 7.6), 50 mM NaCl, and 10 mM MgCl₂ after end-labeling with T4 polynucleotide kinase (Promega) and the radionucleotide [γ -³²P]-ATP (Amersham). Protein-DNA binding assays were performed with 15–25 µg of nuclear extract. To minimize salt on binding, the concentration of salt was adjusted to the same level in all samples. Nonspecific binding was blocked using 1 µg of poly (dI-dC)-poly (dI-dC). The binding medium contains 5% glycerol, 1.0 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM DTT (32). In each reaction 20,000 cpm of radiolabeled probe was included. For Sp1 binding assays, 1.0% (v/v) Nonidet P40 was included. Samples were incubated at room temperature for 20 min. The nuclear protein-³²P-labeled oligonucleotide complex was separated from free ³²P-labeled oligonucleotide by electrophoresis through a 6% native polyacrylamide gel in a running buffer of 0.5× TBE (50 mM Tris, pH 8.0, 45 mM boric acid, 0.5 mM EDTA). After a suitable separation was achieved, the gel was vacuum dried for autoradiography and exposed to Fuji X-ray film exposing at –80°C for 1–2 days.

Assessment of oxidative stress. We used oxidation-sensitive carboxy-H₂DCFDA (C400) and oxidation-insensitive carboxy-DCFDA (C369) fluorescent dyes. The oxidation-insensitive C369 dye was used as a control to ensure that changes in the fluorescence seen with the oxidation-sensitive C400 dye were due to changes in ROS production. The C400 and C369 were freshly dissolved with homogenate buffer and added to liver homogenate in a 96-well plate to achieve a final

TABLE 1. DNA SEQUENCES FOR DOUBLE-STRANDED OLIGONUCLEOTIDES USED IN EMSA

Oligonucleotide	(5' → 3') Nucleotide position	Sequence
Site 3	(–775 to –752)	5'-GCCTAGTTCAAAGCAGTAAGACCC-3'
Site 4	(–739 to –713)	5'-GTTGAAGGTGCTGGCAATTTCCATCAG-3'
Site 5	(–585 to –565)	5'-GTCTTTGGCCTAAAGTCCAAG-3'
Site 6	(–542 to –513)	5'-GGGTTCAAGGCTCCCTGCCAAACTGGCCTC-3'
GATA	(–304 to –277)	5'-GGAGATAGGGACAGTGCCAGCTCTTCAT-3'
Sp1	Commercial sequence	5'-ATTCGATCGGGGCGGGGCGACC-3'

Only the sense strand is shown for each site. These sites were determined by DNase I footprinting on the SMP30 promoter. Site 3, site 4, and site 5 were demonstrated as novel DNA binding sites of nuclear transcription factors. GATA and Sp1 were regarded as contributing to the regulation of SMP30 gene expression (23).

