ANTIOXIDANTS & REDOX SIGNALING
Volume 8, Numbers 3 & 4, 2006

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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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 Ca^{2+}-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 Ca^{2+} homeostasis by enhancing plasma membrane Ca^{2+}-pumping activity (13), the downregulation of SMP30 expression in aged tissue may induce the dysregulation of Ca^{2+} 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 TNFα- 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, TNFα (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 Ca^{2+} 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 Institute of Gerontology. A horseradish peroxide-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-^{32}P]-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 m*M* potassium phosphate buffer containing 1 m*M* EDTA, 1 m*M* *p*-aminobenzamidine, 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 $NaHCO_3$. Oxygen tensions in the incubator were 140 mmHg (5% CO_2, 20% O_2, 75% N_2; 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 m*M* Tris-HCl, pH 8.0, containing 1.5 m*M* $MgCl_2$, 1 m*M* 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 m*M* Tris-HCl, pH 8.0, containing 50 m*M* KCl, 300 m*M* NaCl, 1 m*M* DTT, 5 μg/ml pepstatin, 5 μg/ml aprotinin, and 10 μ*M* 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 m*M* Tris, pH 7.5, 100 m*M* 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 m*M* Tris-HCl (pH 7.6), 50 m*M* NaCl, and 10 m*M* $MgCl_2$ after end-labeling with T4 polynucleotide kinase (Promega) and the radionucleotide [γ-^{32}P]-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 m*M* Tris-HCl (pH 7.5), 50 m*M* NaCl, 1 m*M* EDTA, 1 m*M* 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-^{32}P-labeled oligonucleotide complex was separated from free ^{32}P-labeled oligonucleotide by electrophoresis through a 6% native polyacrylamide gel in a running buffer of 0.5× TBE (50 m*M* Tris, pH 8.0, 45 m*M* boric acid, 0.5 m*M* 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-H_2DCFDA (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 RS 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′-ATTCGATCGGGGCGGGGCGAGC-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).

concentration of 25 μ*M*. The change in fluorescence intensity was monitored at every 5 min for 30 min (Genius, Tecan Instruments, Ex 485 nm/Em 530 nm) (21).

Protein assay. The protein concentration was measured using Lowry's method (25) or bicinchoninic acid method based on bovine serum albumin as the standard.

Statistical analysis. Results were analyzed statistically using one factor ANOVA. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Effects of age-related oxidative stress and CR on SMP30 gene expression

To investigate effects of age-related oxidative stress on SMP30 gene expression in aged rat liver, SMP30 protein levels were measured using Western blot analysis. Further, to assess the overall age-related oxidative status, total reactive species (RS) levels were measured with a carboxy-H_2DCFDA probe (C400) in liver homogenates. Results in Figure 1A show changes in RS levels with age in the AL group, most notably in 24-month-old rats. However, CR rats showed consistently reduced RS levels compared with their AL counterparts at all ages studied. The mean fluorescent intensity of nonoxidation sensitive dye (C369) did not change during aging, meaning that age and CR were closely related to the levels of oxidative stress. The results shown in Figure 1B indicate a significant decrease in the cytoplasmic SMP30 protein level in livers from the AL-fed rats, while the livers of the CR rats were shown to have slightly decreased levels of SMP30 protein throughout life. The same tendency of reduced decreases in SMP30 by CR during aging was observed in nuclear SMP30 with cytosol. Thus, a decline in SMP30 gene expression with age was counteracted by the antioxidative actions of CR.

To further investigate the effect of oxidative stress on SMP30, a potent of oxidative inducer *t*-BHP was injected to

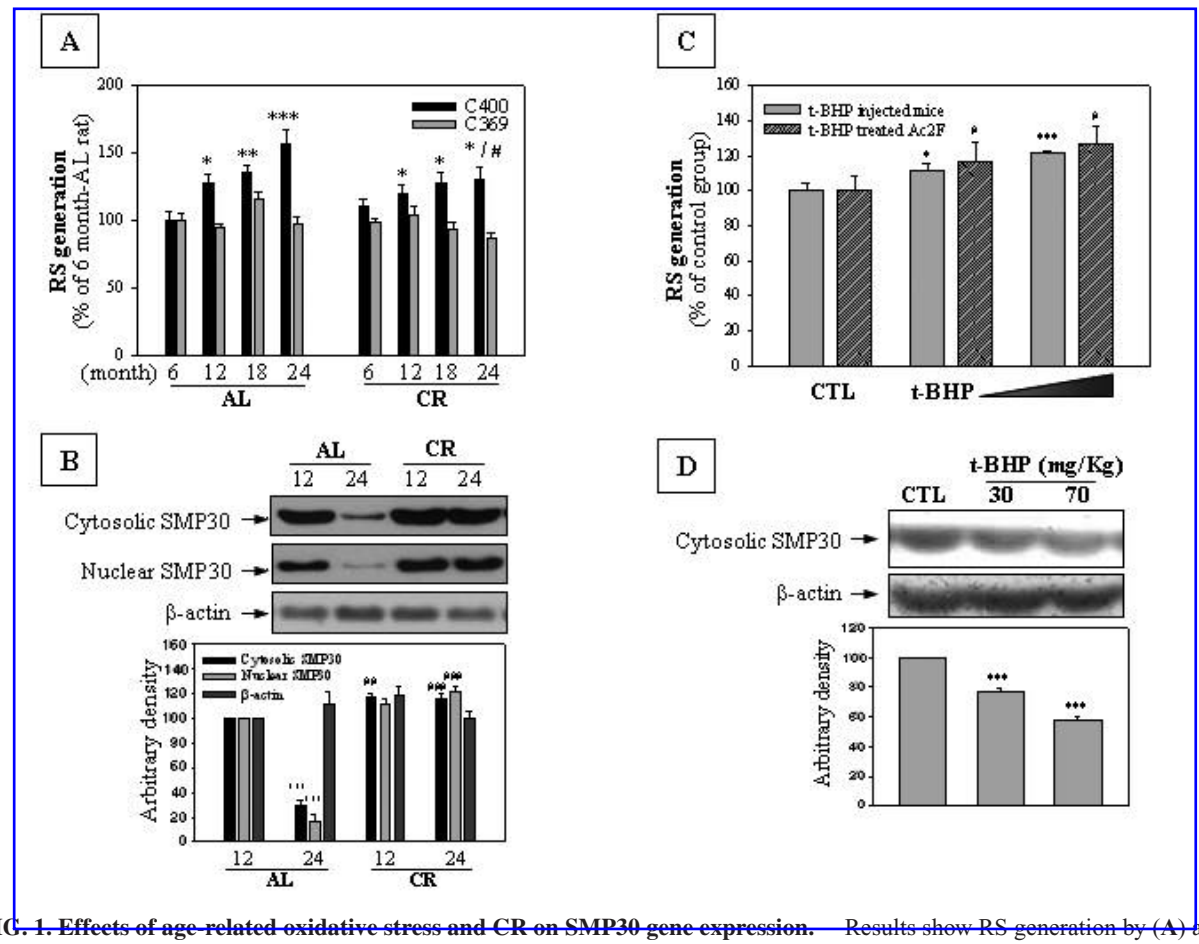

FIG. 1. Effects of age-related oxidative stress and CR on SMP30 gene expression. Results show RS generation by (**A**) and one representative blot of SMP30 by Western blot (**B**) was shown in AL and CR rats during aging. (**C**) shows RS generation by *t*-BHP induced oxidative stress, t-BHP (30 mg and 70 mg/kg) injected mice liver homogenate and *t*-BHP (10 and 30 μ*M*) treated Ac2F liver cell lysate. (**D**) shows one representative blot of SMP30 by Western blot. β-Actin was used for an equal loading control. The experiment was done in triplicate. AL, *ad libitum* group; CR, calorie restricted group; RS, reactive species; CTL, vehicle-treated control group. As statistical significance, results of one factor ANOVA: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ versus 6-month-old-rats of AL group or *t*-BHP untreated group, and $\#p < 0.05$ *vs*. the same aged AL rats, respectively.

male ICR mice. Also, cultured Ac2F liver cells were treated with *t*-BHP. Results in Figures 1C and 1D show that the decreased SMP30 expression correlated dose-dependently with *t*-BHP-induced oxidative stress.

Effect of age-related oxidative stress on transcriptional regulation of SMP30

Tests were performed to ascertain whether the downregulation of SMP30 may be due to reduced transcriptional regulation. Since the SMP30 promoter region contains many transcription binding sites, including Sp1, GATA factor, and newly discovered novel transcription binding sites, they were also examined.

Figure 2 shows the binding sites of transcription factors. To examine how aging and CR can affect DNA binding activities, EMSA was performed using rat nuclear protein. Sp1 and GATA factors were compared against the DNA binding activities at sites that are redox-sensitive transcription factors. Furthermore, as shown in Figure 2A, DNA binding activities at sites 3 and 5 decreased more significantly with age compared to sites 4 and 6. Figure 2B on the DNA binding activities of GATA and redox sensitive Sp1 shows a comparison with the effect of age and CR among sites 3, 4, 5, and 6. However, the DNA binding activities of GATA and Sp1 showed no significant age-related changes in relative to the DNA binding activities of sites 3 and 5, raising the possible implication of site 3 and 5. The sensitivity of SMP30 transcription to oxidative stress was detected by the LPS-induced treatment, which causes oxidative stress. We used EMSA to explore the exaggerated effect of LPS-induced oxidative stress on DNA binding activity during aging. As shown in Figure 2C, although the DNA binding activities of sites 3 and 5 decreased in the old group, the decrease in the LPS-challenged group was much more severe during aging. These results showed that transcriptional binding activities of SMP30 significantly decreased by an aggravated response from age-related and LPS-induced oxidative stress. However, as Figure 2C shows, changes in the DNA binding activities of sites 4 and 6 by the LPS-challenge showed no clear decreases. GATA was shown as comparing it to unidentified transcription factors. Therefore, results indicate that DNA binding activities of sites 3 and 5 are redox-sensitive.

Effects of oxidant t-BHP and antioxidant NAC on DNA binding activities of unidentified transcription factors in vitro

Binding activities at sites 3 and 5 are influenced by increased RS during aging (Figs. 2A and 2C). To verify more directly the effect of oxidative stress on sites 3 and 5, *t*-BHP treatment was used in nuclear protein to observe any changes in DNA binding activities of unidentified factors *in vitro*. As Figure 3A shows, DNA binding activities at sites 3 and 5 decreased in a dose-dependent manner with *t*-BHP treatment, but sites 4 and 6 and GATA changed little (Fig. 3B). Thus, results show that sites 3 and 5 seem sensitive to oxidative stress. To further explore the finding of the *t*-BHP effect, we used an inhibitor of oxidative stress, the potent antioxidant, NAC, to block the *t*-BHP-modulated binding activities of sites 3 and 5, confirming data in Figure 3C. Results in Figure 3C showed that antioxidant, NAC treatment dose-dependently ameliorates the decreased binding activities of sites 3 and 5.

Evidence for the effects of oxidant t-*BHP and ERK inhibitor in a cultured cell system*

To verify effects of oxidative stress and an antioxidant on SMP30 gene expression and the DNA binding activities of sites 3 and 5, we compared the actions of *t*-BHP and NAC on DNA binding activities in a cell nuclear extract. Cells were incubated with *t*-BHP and NAC during 1 h for separation of nuclear fraction and 5 h for separation of cytoplasmic fraction, respectively. Data in Figure 4A shows that decreased SMP30 expression by *t*-BHP was blunted by the addition of NAC. DNA binding activity of sites 3 and 5 showed a similar tendency in the expression of SMP30. In view of these results, age-related oxidative stress is responsible for the decline in SMP30 with age.

To determine the effects of oxidative stress and potentiality the phosphorylation of NF-κB and MAPK on SMP30 gene expression and DNA binding activities of sites 3 and 5, treatments with kinase inhibitors and *t*-BHP were tested. Cells were incubated with *t*-BHP and several kinase inhibitors, Bay 11–7085, PD098059, SB203580, and Wortmannin (specific inhibitors for NF-κB, ERK, MAPK, and phosphatidylinositol 3-kinase (PI_3K), respectively) for 1 h to separate the nuclear fraction or for 5 h to separate the cytoplasmic fraction. Figure 5A reveals that SMP30 expression decreased from the *t*-BHP treatment, whereas the cells treated with the ERK specific inhibitor, PD098059, recovered significantly from the decreased SMP30 brought about by *t*-BHP. As shown in Figure 5B, DNA binding activity at sites 3 and 5 decreased with *t*-BHP treatment, and was reversed with PD098059 treatment. These results revealed that the oxidative stress-induced decrease of DNA binding activity at sites 3 and 5 are likely regulated by the ERK pathway.

DISCUSSION

The current study was undertaken to elucidate the transcriptional regulation of SMP30 under increased oxidative stress during aging. Our data showed that the decline in the binding activity is due mainly to an inability of sites 3 and 5 to bind with nuclear transcriptional factors, which are influenced by redox status. Our findings also suggested that the declined transcriptional regulation of SMP30 is regulated by the ERK pathway. Based on these new revelations, we constructed a schematic model of SMP30 in Figure 5.

Supakar *et al.* (32) reported that an −800 bp upstream SMP30 promoter region and several novel DNA binding sites of transcription factors. To characterize the relation between senescent-related oxidative stress and SMP30 gene expression as a senescence marker protein, we studied the DNA binding activities of unknown transcription factors. The results from our current study affirmed the importance of the binding activities at sites 3 and 5 in relation to the transcriptional regulation of SMP30 as revealed by EMSA data. The results showing no correlation between GATA DNA binding

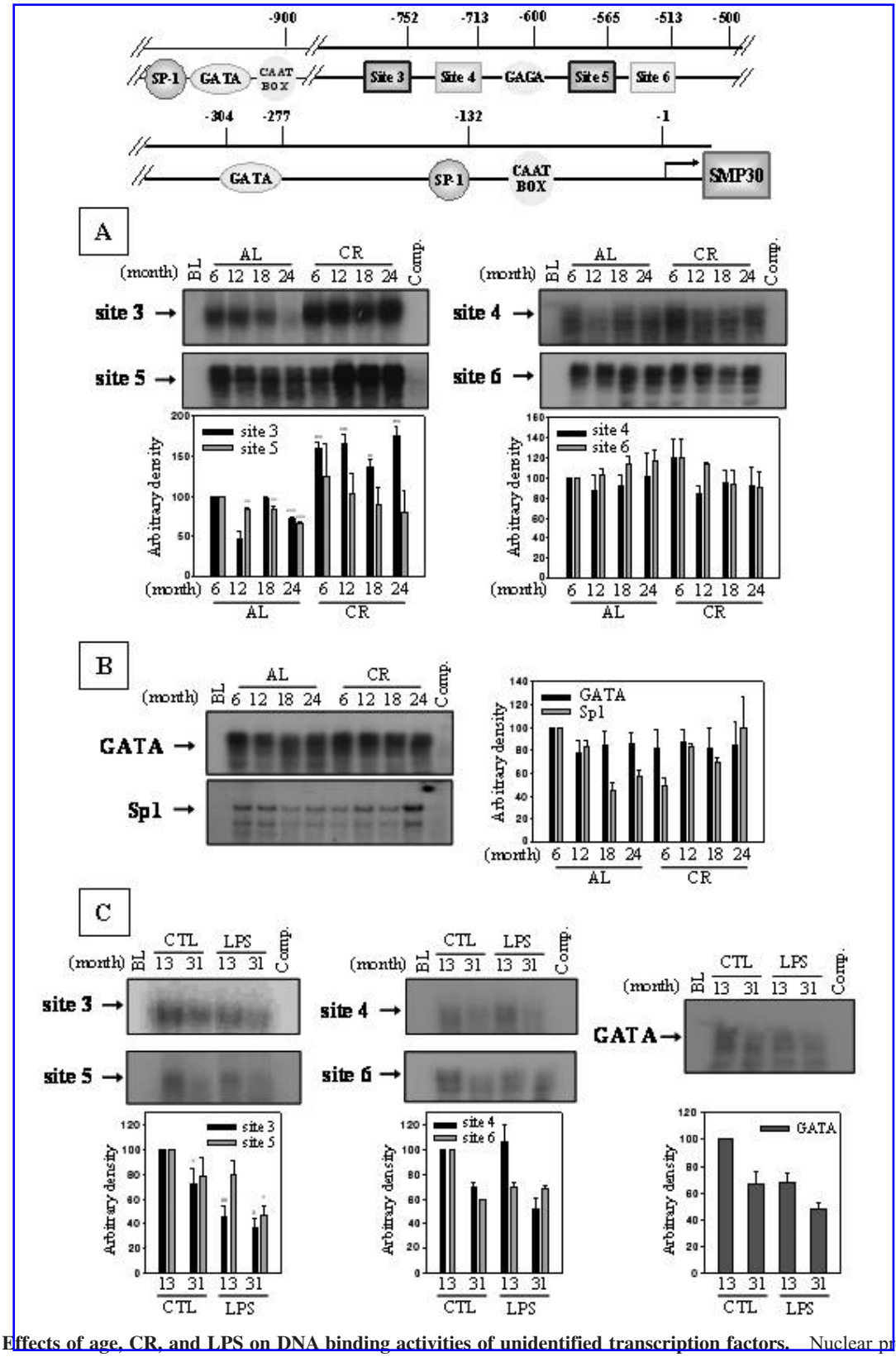

FIG. 2. Effects of age, CR, and LPS on DNA binding activities of unidentified transcription factors. Nuclear protein extracts (15 μg protein) of each group were incubated with ^{32}P-end-labeled probes containing a binding site (see Table 1). Binding activities of unidentified transcription factors are shown in (**A**) and announced transcription factor, GATA and Sp1 in (**B**) compared with unidentified transcription factors. (**C**) Nuclear extracts from rat liver administrated with LPS (5 mg/kg) at 13 and 31 months old were resolved by EMSA. In the present hypothetical study, the most obvious change was shown at sites 3 and 5. AL, *ad libitum* group; CR, calorie restricted group; CTL, vehicle-treated control group; LPS, LPS-injected group; BL, blank as no addition of nuclear fraction; comp., competitive confirmation with content of excess unlabeled oligonucleotides (200-fold competitor). As statistical significance, results of one factor ANOVA: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ *vs*. 6-month-old-rats of AL group or 13-month-old rats of CTL group; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ versus the same aged AL rats or CTL group, respectively.

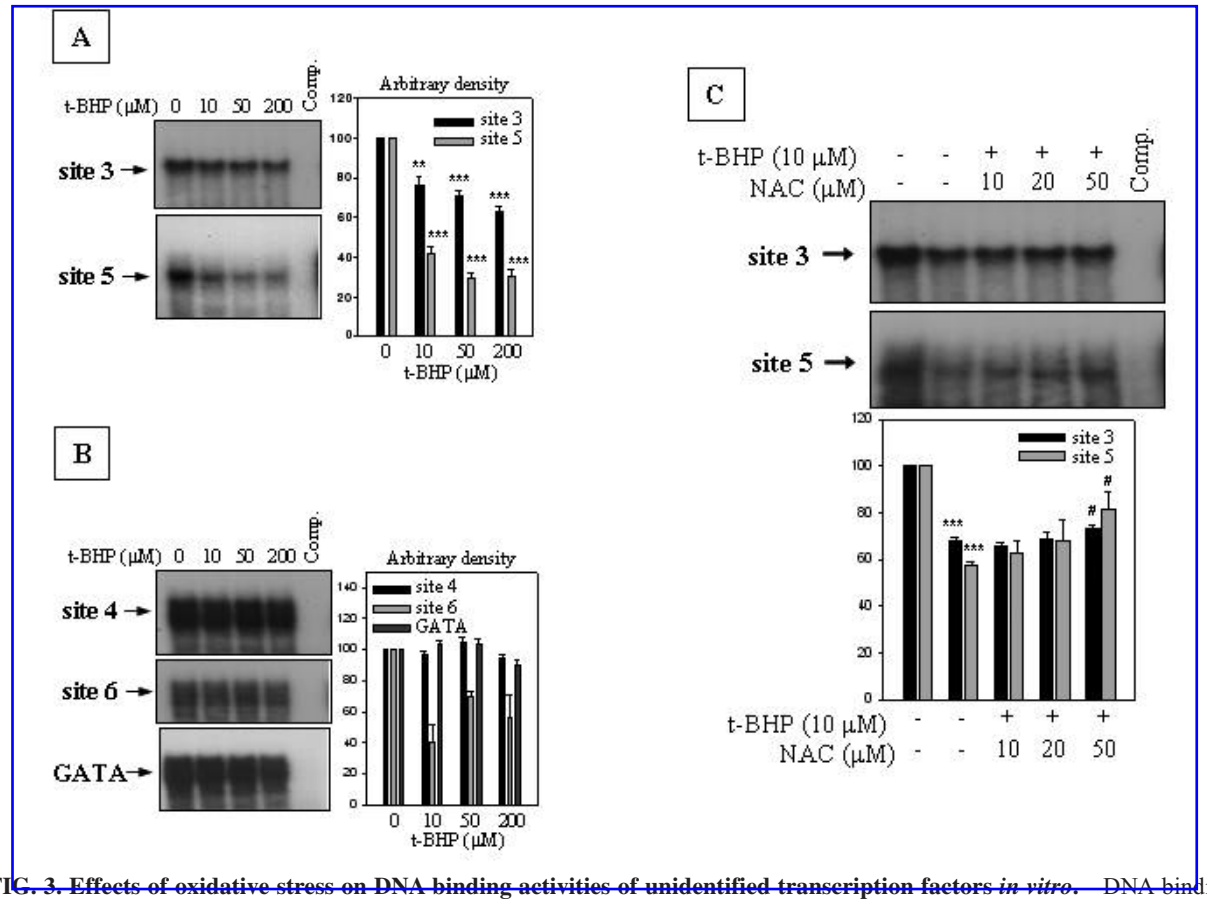

FIG. 3. Effects of oxidative stress on DNA binding activities of unidentified transcription factors *in vitro*. DNA binding activities are shown of liver nuclear protein (25 μg protein) inflicted by *t*-BHP (μ*M*) *in vitro*, using 6-month-old rats (**A**) and (**B**). An additional antioxidant, NAC (10 ~ 50 μ*M*) and oxidant, *t*-BHP (10 μ*M*) were co-treated to sites 3 and 5 (**C**). CTL, vehicle-treated control group; comp., competitive confirmation with content of excess unlabeled oligonucleotides (200-fold competitor). As statistical significance, results of one factor ANOVA: $^{**}p < 0.01$, $^{***}p < 0.001$ *vs*. *t*-BHP untreated group, and $\#p < 0.05$ versus *t*-BHP only treated group, respectively.

activities and aging, compared to activity at sites 3 and 5 indicates that the decreased DNA binding activity at sites 3 and 5 occurs in their promoter regions due to increased oxidative stress. To verify these results, we checked binding activities of the SMP30 promoter region utilizing oxidative stress-promoting, LPS-treated rats. Findings from the LPS treatment that elicits oxidative stress showed suppressed nuclear binding activity and that the antioxidant, NAC, blocked decreased DNA binding activities at sites 3 and 5, further supporting the binding reaction's sensitivity to the redox condition. However, the exact nature of oxidative modifications that influence the binding reaction is yet to be determined.

Redox-based gene expression has emerged as a fundamental regulatory mechanism of several redox-sensitive transcription factors that have been ascribed as apparent redox-sensing activity (24, 30). The functional thiol group of conserved cysteinyl residues in signal proteins account for their redox-sensing properties, which are oxidized and potentially affect redox signaling. Signals are transduced from the cell's surface into the nucleus through phosphorylation and dephosphorylation chain reactions of amino acid residues such as tyrosine and serine/threonine (33). Protein phosphorylation is one of the most fundamental mediators of cell signaling and is redox-sensitive. DNA-binding proteins, like NF-κB, AP-1, and p53, contain reactive thiols in their binding regions, and also are involved in the regulation of the redox status (26, 27). When the cysteine residues of a redox-sensitive transcription factor are phosphorylated, DNA binding activity is upregulated or downregulated by oxidative stress-induced phosphorylation (18).

Our previous work suggested that the downregulation of SMP30 with age is likely due to increased oxidative stress (19). In the current study, decreased SMP30 was further explored with the use of LPS and *t*-BHP challenges, both of which elicit oxidative stress that mimic what might occur with aging. Both *t*-BHP and LPS also are reported to be potent stimuli that induce oxidative stress in various cell culture systems, including hepatocytes, erythrocytes, and fibroblasts (5, 29), and several tissues such as brain, testis, and heart (2, 10). With *t*-BHP and LPS treatments, binding sites 3 and 5 showed significantly decreased binding activity, indicating the redox-sensitive nature of the DNA binding reaction. Results showing downregulated SMP30 by oxidative stress and the counteraction by CR strongly suggested that the change in SMP30 occurring during aging is likely elicited by oxidative stress, influencing the redox-responsive binding at sites 3 and 5.

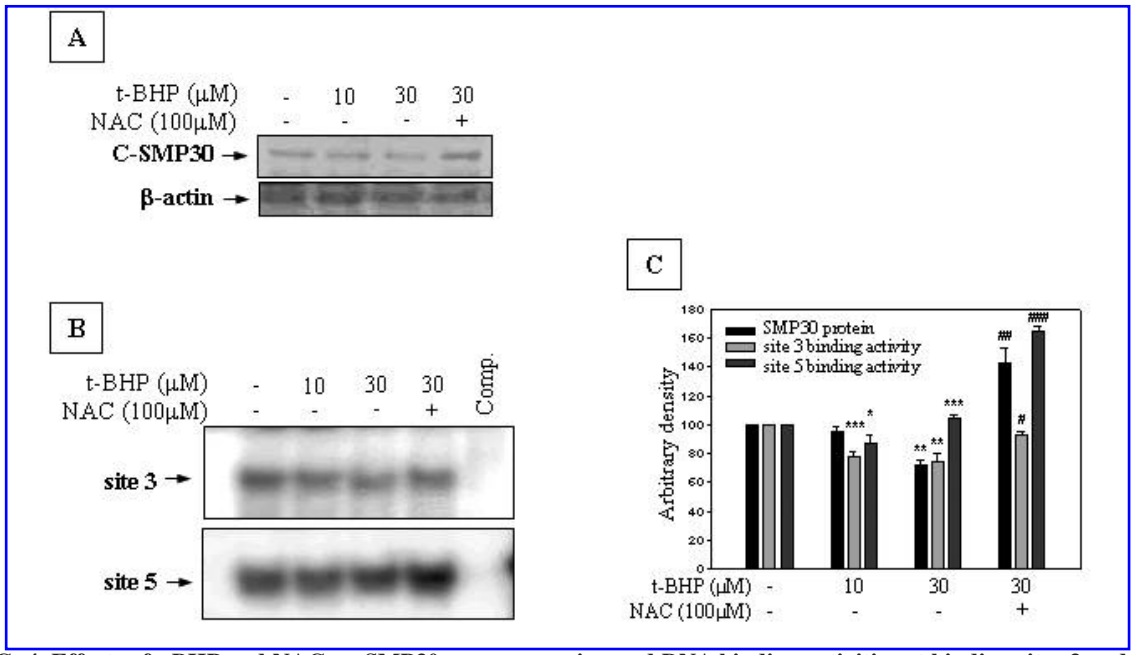

FIG. 4. Effects of *t*-BHP and NAC on SMP30 gene expression and DNA binding activities at binding sites 3 and 5 in cultured Ac2F liver cell. Cells were incubated with *t*-BHP and NAC simultaneously for 1 h or 5 h for fractionation of nuclear or cytoplasmic fraction, respectively. (**A**) SMP30 gene expression in cytosol (50 μg protein) was detected by Western blot. β-actin was used for an equal loading control. (**B**) Binding sites 3 and 5 were detected by EMSA with incubating ^{32}P-end-labeled oligonucleotides and nuclear protein extracts (15 μg protein). (**C**) Densitometric measurements showed effects of *t*-BHP and NAC in (**A**) and (**B**). C-SMP30, cytosolic SMP30; Comp., content of excess unlabeled oligonucleotides (200-fold competitor). As statistical significance, results of one factor ANOVA: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ versus *t*-BHP untreated group, and $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ *vs*. *t*-BHP treated group, respectively.

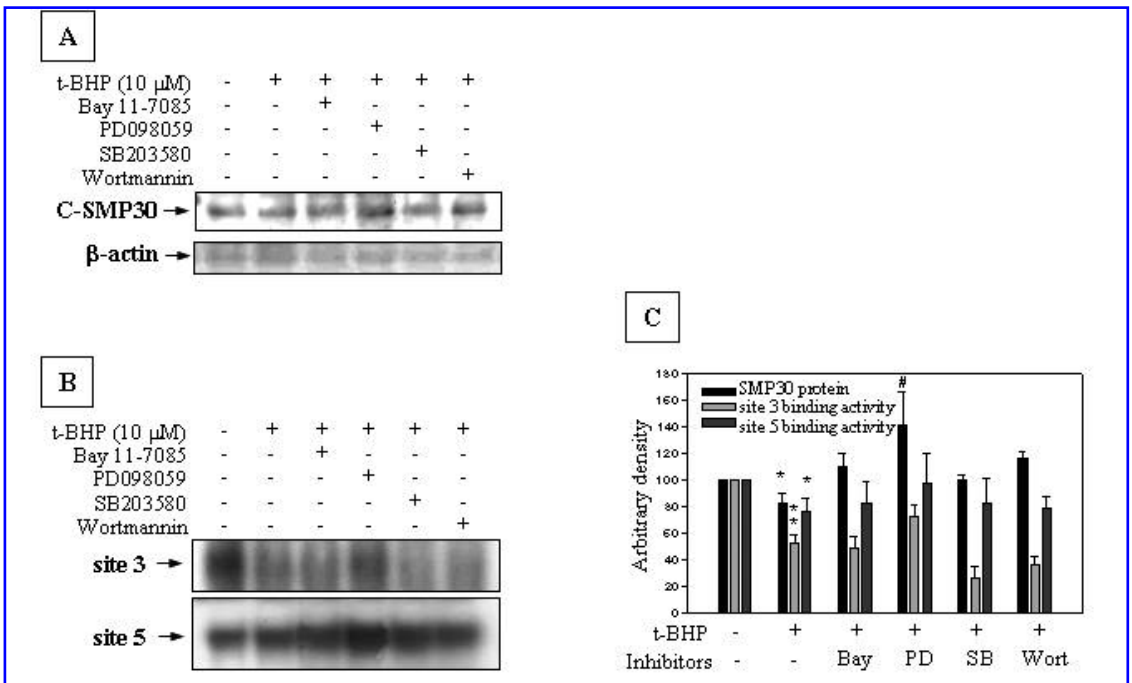

FIG. 5. Effects of PD098059 on SMP30 gene expression and DNA binding activities at binding sites 3 and 5 in cultured cell. Cells were incubated with kinase inhibitors and *t*-BHP simultaneously for 1 h or 5 h for fractionation of nuclear or cytoplasmic fraction, respectively. Bay 11–7085 (2 μ*M*), PD098059 (10 μ*M*), SB203580 (10 μ*M*), and Wortmannin (100 n*M*) were treated for inhibition of specific kinases. Kinase inhibitors were treated 10 min previously, and then *t*-BHP (10 μ*M*) were treated. (**A**) SMP30 gene expression in cytosol (50 μg protein) was detected by Western blot. β-Actin was used for an equal loading control. (**B**) Binding sites 3 and 5 were detected by EMSA with incubating ^{32}P-end-labeled oligonucleotides and nuclear protein extracts (15 μg protein). (**C**) Densitometric measurements showed effects of *t*-BHP and NAC in (**A**) and (**B**). C-SMP30, cytosolic SMP30; Comp., content of excess unlabeled oligonucleotides (200-fold competitor). As statistical significance, results of one factor ANOVA: $*p < 0.05$, $**p < 0.01$ versus *t*-BHP untreated group, and $\#p < 0.05$ versus *t*-BHP treated group, respectively.

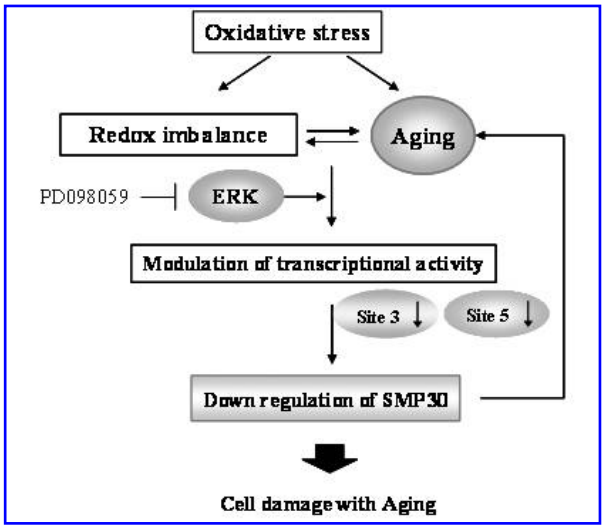

FIG. 6. Potential pathway for SMP30 gene expression regulation via ERK. Age-related oxidative stress leads to redox imbalance, decreasing SMP30 gene expression. Binding sites 3 and 5 that concern redox-sensitive SMP30 transcription are downregulated by oxidative stress and regulated by the ERK pathway. This is a hypothetical scheme for the age-associated decrease in SMP30 by oxidative stress that focuses on binding sites 3 and 5. SMP30 plays an important role as a Ca^{2+} regulating protein, which removes Ca^{2+} from the cytosol across the plasma membrane. In aged tissues, the gene expression of SMP30 is decreased, thereby influencing the alteration of the signaling system and the emergence of age-associated deterioration of cellular function associated with hypoxia, inflammation, and aging (6). *Thin arrow*, present work; *bold arrow*, reported results.

Given the ubiquitous expression of the MAPK pathway, the high degree of evolutionary conservation, and the wide range of cell-surface stimuli that trigger ERK activation, it is not surprising that this signaling module is involved in a vast number of cellular functions, including proliferation, differentiation, survival, migration, and adhesion. The large number of various stimuli that can lead to ERK activation renders ERK an essential signaling cross-road within the cell. Compelling evidence links ERK activation to cell degeneration and even cell death by exerting noxious effects leading to excitotoxicity or oxidative stress by increasing reactive oxygen species (ROS) (3). It was suggested that the kinetics and duration of ERK activation may determine whether downstream targets will trigger beneficial or detrimental effects on cells (e.g., a prolonged activation of ERK following ROS elevation). Additionally, cellular senescence is characterized by the ERK pathway in its participation in cell death due to oxidative stress and calcium dysregulation (34). Investigators report that senescent cells accompany the activation of the ERK pathway, which is consistent with reports that show a defective accumulation of ERK1/2 entered senescence after serial passage in the cell (3, 34).

To date, finding a link between the ERK pathway and SMP30 has not been reported. In this study, we showed that the inactivation of signal pathway ERK by the inhibitor, PD098059 may rescue the cell from the oxidative stress-induced decrease in SMP30 gene expression via binding sites 3 and 5. These molecular modulations revealed the decline of SMP30 gene expression through the regulation of the ERK pathway. Our study showed that the downregulated transcriptional expression of SMP30 and the downregulated SMP30 during aging are likely caused by decreased binding activity of transcription factors at sites 3 and 5 due to increased age-related oxidative stress. Our study further showed that the downregulation of both SMP30 gene expression and its transcriptional activity are implicated in the ERK pathway.

ACKNOWLEDGMENTS

This study was supported by a grant of the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (02-PJ1-PG3–20803–0004). We are grateful to the 'Aging Tissue Bank' for supplying aged tissue.

ABBREVIATIONS

ANOVA, analysis of variance; bp, base pair; C-SMP30, cytosolic senescence marker protein-30; CR, calorie restriction; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal regulated kinase; GATA, transcription factor GATA binding protein; JNK, c-Jun-N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen activated protein kinase; NAC, *N*-acetylcysteine; NF-κB, nuclear factor κB; PVDF, polyvinylidene fluoride; ROS, reactive oxygen species; SMP30, senescence marker protein-30; Sp1, transcription factor stimulating protein, *t*-BHP, *tert*-butylhydroperoxide; TNFα, tumor necrosis factor α.

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Received after final revision September 12, 2005; accepted September 12, 2005.

This article has been cited by:

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