STEROL REGULATORY ELEMENT BINDING PROTEIN (SREBP)-1-MEDIATED LIPOGENESIS IS INVOLVED IN CELL SENESCENCE

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Increased cell mass is one of the characteristics of senescent cells, but this event has not been clearly defined. When subcellular organellar mass was estimated with organelle-specific fluorescence dyes, we observed that most membranous organelles progressively increase in mass during cell senescence. This increase was accompanied by an increase in membrane lipids and augmented expression of lipogenic enzymes, such as fatty acid synthase (FAS), ATP citrate lyase (ACL), and acetyl-CoA carboxylase. The mature form of sterol regulatory element binding protein (SREBP)-1 was also elevated. Increased expression of these lipogenic effectors was further observed in the liver tissues of aging Fischer 344 rats. Ectopic expression of mature form of SREBP-1 in both Chang cells and primary young human diploid fibroblasts (HDFs) was enough to induce senescence. Blocking lipogenesis with FAS inhibitors (cerulenin and C75) and via siRNA-mediated silencing of SREBP-1 and ACL significantly attenuated H2O2-induced senescence. Finally, old HDFs were effectively reversed to young-like cells by challenging with FAS inhibitors. Our results suggest that enhanced lipogenesis is not only a common event, but also critically involved in senescence via SREBP-1 induction, thereby contributing to the increase in organelle mass (as a part of cell mass), a novel indicator of senescence.

Enlarged cell morphology is a typical characteristic of senescent cells, reflecting increased cell mass (1-3). However, the detailed components of the mass are not clearly defined, and whether the increase in mass is due to the increase of any specific cellular compartment(s), or all, is unknown. Until now, the lysosome has been the most recognized organelle increased in both senescent and aged cells (4, 5). However, senescent lysosomes are filled with indigestible autofluorescent pigments, such as lipofuscin, and displaying non-functionality (6). Recently, an increase in mitochondrial mass has been reported in stress-induced senescence (7) and replicative senescence (8, 9), which was generally explained to be the result of its compensatory biogenesis to cope with the persistent functional defects of mitochondria (10, 11). An increase in mitochondrial mass is further augmented by the inability of the lysosome to remove damaged mitochondria, thereby allowing the damaged mitochondria to continuously generate hazardous reactive oxygen species (ROS). This sequence suggests that most senescent mitochondria are also not functionally intact. The functional deterioration of mitochondrial respiration accompanied by a dramatic increase in their mass during cell senescence and aging has often been found, supporting the hypothesis (7, 12). The close link between the accumulated non-functional organelles, mitochondria and lysosomes, yields the “mitochondrial-lysosomal axis theory of aging” (13, 14). This theory sufficiently supports the generation of persistent oxidative stress during the progression of senescence. Analogous to this theory, the inevitable increase in non-functional organelles due to the combined actions of the uncontrolled ROS generator (mitochondria) and the non-functional eradicator (lysosome) is likely not restricted to mitochondria, but may be applied to all...
subcellular components. Therefore, whether all cellular organelles, especially membranous ones, are also under the compensatory enhancement of their biosynthesis and, if so, whether these features are genuinely involved in the progression of cell senescence should be evaluated. Monitoring the biogenetic activity of each organelle is not simple; however, lipid biosynthesis is a critical element for the biogenesis of all membranous organelles and may be a plausible barometer of overall membranous organelle biogenesis.

Lipid biosynthesis, called lipogenesis, includes fatty acid synthesis and the subsequent synthesis of triglycerides and membrane phosphoglycerides. Lipogenesis is activated during cell growth and proliferation to provide membranous components for subcellular organelle formation (15, 16). Lipogenesis comprises multiple sequential steps governed by several key lipogenic enzymes, including ATP citrate lyase (ACL), acetyl-CoA carboxylase (ACC), and fatty acid synthase (FAS) (17). ACL converts cytosolic citrate into acetyl-CoA and oxaloacetate, supplying essential metabolites for lipid biosynthesis. ACC catalyzes the irreversible carboxylation of acetyl-CoA to produce malonyl-CoA, and FAS subsequently condenses acetyl-CoA and malonyl-CoA to generate long-chain fatty acids. Interestingly, the expression of these lipogenic enzymes is controlled by the common lipogenic transcription factors, sterol regulatory element-binding proteins (SREBPs). Three SREBP isoforms (SREBP-1a, -1c, and -2) have been identified. SREBP-1a and -1c are alternative spliced forms transcribed from two distinct promoters on the same gene whereas SREBP-2 is encoded from a different gene. SREBP-1 plays a greater role in regulating fatty acid synthesis than cholesterol synthesis, whereas SREBP-2 is relatively selective in transcriptionally activating genes involved in cholesterol biosynthesis (18, 19). SREBPs are initially bound to the rough endoplasmic reticulum membrane but, upon activation, are cleaved to liberate the N-terminal portion containing a basic helix-loop-helix leucine zipper (bHLH-Zip) domain. The cleaved active fragment enters the nucleus, where it binds to specific sterol response elements within the promoter regions of the lipogenic genes and activates their transcription (17, 18). Therefore, SREBP activation can be used as a good indicator of the lipogenesis status of the cell. In addition, SREBP is the best target for controlling cellular lipogenesis and exploring the relationship between lipogenesis and senescence.

In this study, we show that all subcellular organelles are generally increased with membrane lipids in both stress-induced senescence and replicative senescence. In addition, we demonstrate that lipid biosynthesis is directly involved in senescence-associated organelle biogenesis via SREBP-1 activation. These results suggest that SREBP-1-mediated lipogenesis is an important modulator of senescence and provide new insight into the metabolic background of aging and aging-related pathogenesis. The overall organelle increase is also indicated as a novel indicator of senescence.

**Experimental procedures**

*Cell culture, generation of cell senescence, cell growth rate -* Chang cells were purchased from the American Type Culture Collection (Rockville, MD, USA) and were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS, GIBCO, Grand Island, NY) and antibiotics at 37°C in a humidified incubator with 5% CO2. Chang cells, immortalized normal human hepatocytes, were chosen to understand metabolic backgrounds related to stress-induced senescence due to their homogenous replicative capacity and strong metabolic properties. To generate stress-induced senescence, Chang cells were exposed to 200 µM DFO or H2O2 for the indicated periods. For replicative senescence, the primary HDFs were generously provided by Dr. Lim (Ajou University, South Korea) (20). Confluent cells were evenly transferred into two new dishes and the cells were cultured until getting confluent again to generate one population doubling. The numbers of population doublings (PD, number) as well as the doubling time (DT, days) were continuously counted. Young and old cells used in the present experiments were defined as the HDF with a doubling time of around 24 hours and over 1 week, respectively.

To evaluate cellular growth rate, cells were harvested by trypsinization and counted with a hemocytometer after staining with 0.4% (w/v)
trypan blue (GIBCO) to exclude dead cells. No significant dead cells were observed during both stress-induced and replicative senescence. Total cell number of trypan blue-negative live cells was obtained upon completion of the experiments.

Estimation and visualization of subcellular organellar mass - To estimate cellular organellar mass, cells were stained with organelle-specific fluorescence dyes according to manufacturer’s instruction. Briefly, proper concentrations and incubation times for each organelle were first determined. Mitotracker Red CMXRos (100 nM, M7512, Molecular Probes, Eugene, OR), Lyso Tracker Green DND-26 (1 µM, L7526, Molecular Probes), and ER Tracker Blue-White DPX (1 µM, E-12353, Molecular Probes) were applied for 30 min at 37°C. Especially, to stain Golgi body, cells were incubated for 30 minutes at 4°C with 5 µM BODIPY C5-Ceramide (D3521, Molecular Probes) after pre-conjugating the dye with BSA and further incubated in fresh medium at 37°C for 30 minutes. After staining cells with the dyes, cells were harvested in phosphate-buffered saline (PBS) and their fluorescence intensities were analyzed by flow cytometry (FACSCanto™ II, Becton Dickinson Corp., San Jose, CA). Subcellular organellar masses were represented as mean values of arbitrary fluorescence unit of 10,000 cells.

To confirm specific staining of subcellular organelles with the dyes, fluorescence images of the stained cells were visualized by Axiosvert 200M with a cooled AxioCam HRm operated by the Axiosvision 4.8 software (Carl Zeiss AG, Gottingen, Germany). Nuclear membrane mass was represented by nucleus area (µm²) measured from the nuclear fluorescence images of the Hoechst 33258-stained cells. Cytoplasmic membrane mass was represented by cell area (µm²) measured from phase-contrast images of the cells. Nuclear or cytoplasmic area was calculated automatically by Axiosvision 4.8 software (Carl Zeiss AG).

Determination of intracellular ROS, and cell size and granularity - To estimate intracellular ROS level, cells were stained with 10 µM 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA, Molecular probes, Eugene, OR, USA) for 15 min at 37°C before performing flow cytometric analysis with FACSCanto™ II (Becton Dickinson Corp.). Cell size and granularity were evaluated by analyzing forward scatter (FSC) and side scatter (SSC) of the stained cells, respectively, as previously described (21).

Thin layer chromatographic analysis of cellular lipids - To analyze cellular lipid profiles, cellular lipids were extracted as described by Folch (22) with slight modification. Briefly, the indicated numbers of cells were harvested into glass tubes and mixed with 1 ml of chloroform:methanol (2:1). After vortexing, H2O (500 µl) was added into the tubes and centrifuged at 1500 rpm for 15 minutes. Lower chloroform phase was collected and backwashed by adding methanol (300 µl) and H2O (300 µl). After removing the top layer, chloroform phase were concentrated by evaporating chloroform under N2 gas and loaded onto TLC plate (No. 1.05721.0001, 20 x 20, silica-coated, Merk, Darmstadt, Germany). To separate the cellular lipids, we used the Kupke and Zeugner’s method (23) with a slight modification. Briefly, the chromatogram was first developed in a saturated chamber with solvent I (chloroform:methanol:H2O = 65:30:5) to allow polar-lipids being separated. The solvent front was allowed to migrate approximately 10 cm from the bottom of the plate. After evaporating the solvents, the non-polar lipids were separated by developing the chromatogram with the solvent II (n-hexane:diethyl ether:acetic acid = 80:20:1.5) up to 1 cm from the top of the plate. The plates were dried under a stream of air and sprayed with primulin (Sigma, St. Louis, MO) solution [50 µg/ml in acetone:water (80:20)]. The plates were dried again and fluorescent lipid spots were scanned on UV transiluminator (SL-20, Seoulin Scientific Co., Seoul, Korea) equipped with camera (PC1210, Canon Inc., Japan). The densities of lipid spots were estimated by using Gel-Pro Analyzer 4.0 software (Media Cybernetics, Silver Spring, MD). Standard lipids were obtained from Sigma (St. Louis, MO) as follows: cholesteryl palmitate (CP: C6072), palmitate (PA: P5586), cholesterol (CL: C8667), cardiolipin (CA: C0563), 1,2-Diacyl-sn-glycero-3-phospho-L-serine (PS: P7769), L-α-Lyso phosphatidylcholine (PC: L4129), and 3-sn-Lyso phosphatidylethanolamine (PE: L4754).
Western Blot Analysis and Antibodies - Cells were washed twice with PBS and lysed with lysis buffer [50 mM Tris-Cl, pH 7.5, 0.1 M NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 10 µg/ml each of aprotinin and leupeptin, and 1 mM PMSF]. A portion (30 µg) of the lysate was applied to western blot analysis as previously described (Yoon et al, 2006). Antibodies against SREBP1 (cat. 557036) and SREBP2 (cat. 557037) were purchased from BD Biosciences (San Jose, CA) and antibody for α-tubulin (Ab-1) was from Oncogene (Boston, MA). Antibodies against FAS, ACC and ACL were kindly provided by Prof. Kyung-Sup Kim (Yonsei University, Seoul, Korea). The expression level of each protein was estimated by comparing the band intensities exposed on X-ray film by using Gel-Pro Analyzer 4.0 software.

Preparation and infection of recombinant adenovirus of mature form of SREBP-1 - The recombinant mature SREBP-1 adenovirus (admSREBP1) was prepared as described previously (24). Briefly, the cDNA encoding rat SREBP-1 (1-403 amino acids, a mature form) was cloned into pAd-YC2 shuttle vector. For homologous recombination, pAd-YC2 shuttle vector (5 µg) and a rescue vector, pJM17 (5 µg), were cotransfected into human embryonic kidney 293 cells. Pure plaques were purified and propagated in 293 cells with screening by PCR for the cloned cDNA region. Then, the recombinants were amplified in 293 cells and further isolated using CsCl2 (Sigma). The preparations were collected and desalted, and titers were determined by the measurement of plaque counts. To over-express mature form of SREBP-1(mSREBP1) with the recombinant adenovirus (admSREBP1), approximately 1x10^4 Chang cells or young HDFs (PD16) were seeded on 12 well plates and infected with admSREBP1 at the indicated titer for 12 h at 37°C. The stain was visible 12 h after incubation at 37°C. By counting the numbers of the blue and total cells using Image J software (NIH, Bethesda, MA), percentage of the SA-β-gal positive cells was obtained to estimate the degree of senescence-associated cells.

Senescence associated β-galactosidase (SA-β-gal) assay - SA-β-gal activity was assayed at pH 6.0 as described by Dimri et al., (25) with a slight modification. Briefly, cells were washed twice with PBS (phosphate buffered saline), fixed to plates by 3.5% formaldehyde for 5 min, and then incubated overnight in freshly prepared staining solution [40 mM citrate-phosphate buffer, pH 6.0, containing 1 mg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; Sigma, St. Louis, MO, USA), 5mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM MgCl2].

RESULTS

Overall Mass Increases in Membranous Subcellular Organelles of Senescent Cells. First, we examined changes in mass of all cellular organelles, especially membranous ones, in stress-induced senescence triggered by deferoxamine (DFO) and H2O2 as previously described (7, 26, 27). Subcellular organelles were stained with organelle-specific fluorescent dyes and their masses were estimated by flow cytometric analysis (Fig. 1) after confirming their specificities by visualizing with fluorescence microscopy (Supplementary Fig. S1). Unexpectedly, increased organelle mass was observed not only in lysosomes and mitochondria, but also in other organelles, such as the endoplasmic reticulum and Golgi. Whether the number of organelles was increased or whether their individual volumes enlarged without changes in number was difficult to

Introduction of siRNA into cells - Oligonucleotides for SREBP1 siRNA #1 (5’-GGCCGAAGGCGAGCAAGATT-3’), SREBP1 siRNA #2 (5’-CCGAAAGGCGAGAAAGATT-3’), ACL siRNA #1 (5’-CAAAUAUGUGAGUAGUATT-3’), ACL siRNA #2 (5’-UGAAAGUCUGUGUCUATT-3’) and negative control siRNA (5’-UAGCGACUAAACACAUCAA-3’) were produced by Samchully Pharm.Co. (Seoul, Korea). siRNA duplexes were introduced into cells using Oligofectamine (Invitrogen, Grand Island, NY, USA) according to the manufacturer’s instruction.
clarify, one clear finding was that the total membranous portion of the specified organelle within a single cell was increased. In the case of the nucleus, senescent cells developed either a single giant nucleus or highly conjugated multinuclei. To estimate the membranous portion of the nucleus, we measured its single-sectioned area after staining with Hoechst 33258. Regardless of the morphological diversity, the nuclear area of most senescent cells was significantly increased compared to control cells, suggesting a marked increase in nuclear membrane (Fig. 1B). Increased cytoplasmic membrane was also identified using an analogous approach (Fig. 1C). Moreover, similar results of overall mass increases in membranous organelles were obtained during the replicative senescence of primary human diploid fibroblasts (HDFs) which is the best representative model of cell senescence (Fig. 1, D and E). These results indicate that the mass of all subcellular membranous organelles is commonly increased in senescence, contributing to the increase in cell mass. The results also suggest that membrane lipids, major components of the organelles, may increase for this phenomenon.

Increase in Membrane Lipid Content and Lipogenesis in Senescence. Biological membranes are complex structures with up to 70% of their composition being membrane lipids. To measure membrane lipid content, total cellular lipids were extracted and separated by TLC (Fig. 2A(a)). Total lipids (sum of the lipids indicated as 1-5 in Fig. 2A) increased roughly 3-fold at 2 days and 4-fold at 3 days after exposure to DFO. Potential membrane lipids (sum of the lipids indicated as 2-5 in Fig. 2A) positioned with similar retardation as the standard membrane lipids, such as phosphatidyl ethanolamine (PE), phosphatidyl serine (PS), cardiolipin (CA), and cholesterol (CL), amounted to approximately 89% of the total lipids in control cells, and obviously increased to 95.5% in DFO-induced senescent cells while the potential storage lipid (indicated as 1 in Fig. 2A) with similar retardation as cholesteryl palmitate (CP) decreased (Fig. 2A(b)). Increases in total lipid content and the membrane lipid portion were also observed in H2O2-induced senescent cells (Supplementary Fig. S2A). Undoubtedly, old HDFs were found to have increased total lipids (2.4-fold) and an increased portion of membrane lipids (96.11% in young, 98.4% in old; Fig. 3A). These results indicate that an increase in cellular lipids, especially membrane lipids, can also be recognized as a common characteristic of senescence, supporting the increase in organellar mass.

Next, we investigated whether the increase in membrane lipids results from the activation of lipid biosynthesis. We monitored the expression level of mature form of SREBP-1, which is known to be a master regulator of the transcription of diverse lipogenic enzymes (28). As expected, the active mature form of SREBP-1 was clearly increased in DFO-induced senescence (Fig. 2B). The protein levels of SREBP-1 target enzymes FAS, ACC, and ACL were also monitored. When the enzyme levels were normalized to tubulin, they reached a maximum at 24 h (Fig 2B(b)) and then slightly decreased, which does not correlate with the pattern of total cellular lipid content. We confirmed that cytoplasmic proteins increase is much greater than nuclear proteins increase (data not shown) although nuclear mass also increase (Figure 1B) during cell senescence, implying that overall cytoplasmic mass is the combined result of all the other cytoplasmic organelles except nucleus. Therefore, we normalized the enzyme expression levels to lamin (nuclear protein) to represent the protein level per cell because cellular lipid contents were analyzed based on cell number. In this case, progressive and dramatic increases in the lipogenic enzymes were clearly observed (Fig 2B(c)). Similar results were obtained with H2O2-induced senescence (Supplementary Fig. S2C). This increased expression of lipogenic enzymes and the active form of their master key regulator, SREBP-1c, was further observed in replicative senescence (Fig. 3, B and C) and the aging F344 rat model (Fig. 4), even when normalized to tubulin. Taken together, these results suggest that enhanced lipogenesis via SREBP-1 activation may contribute to the increased cellular lipids of senescence and aging.

Enhanced Lipogenesis is Enough to Induce Cellular Senescence. To determine whether enhanced lipogenesis is genuinely involved in the development of cell senescence, we examined the effect of enhanced lipogenesis using the ectopic expression of active SREBP-1.
We introduced adenovirus harboring the NH₂-terminal fragment (mature form) of rat SREBP-1 into Chang cells. As shown in Fig. 5A, over-expressed mature SREBP-1 (mSREBP1) was sufficient to increase the expression of FAS and ACC without affecting endogenous SREBP-1 or SREBP-2 expression. mRNA levels of all the lipogenic enzymes (FAS, ACC and ACL) were significantly induced by mSREBP1 at the transcriptional level, implying its transcriptional activation (Supplementary Fig. S4, A). This finding implies that mSREBP-1 over-expression can effectively enhance cellular lipogenesis in a cell culture system. Excitedly, this enhanced lipogenesis by mSREBP-1 over-expression alone clearly induced senescence, as shown by an increase in SA-β-gal activity, arrest of cellular growth, increased cell granularity represented by side scattering, and increased cell size represented by forward scattering (Fig. 5, B and C). The induction of senescence was further confirmed by the induction of the p21 and p16 proteins, the inhibitory cell cycle regulators (Fig. 5, A). Similar and more remarkable results by over-expressing SREBP-1 on gain of senescence phenotypes, including SA-β-gal, granularity (SSC) and delayed cell growth in addition to induction of lipogenic enzymes were obtained in young HDFs (Fig. 6 and Supplementary Fig. S4, B). These data provide strong evidence that enhanced lipogenesis, at least by SREBP-1 activation, is sufficient for inducing senescence.

Blocking Lipogenesis Significantly Attenuates Cell Senescence. Next, we investigated the effect of blocking lipogenesis on H₂O₂-induced senescence using siRNA-mediated knockdown of SREBP-1 or ACL expression and pharmacological FAS inhibitors. SREBP-1 knockdown effectively attenuated all senescence phenotypes induced by H₂O₂ (Fig. 7, A and B). Attenuation of the senescence phenotypes correlated well with intracellular ROS levels, suggesting that the enhanced lipogenesis may be tightly linked to cellular oxidative stress (Fig. 7B(d)). The H₂O₂-induced senescence was also diminished by siRNA-mediated ACL knockdown or FAS-specific inhibitors cerulenin and C75 (29, 30) as shown in Fig. 7, C and D.

Finally, we investigated whether the inhibition of lipogenesis can reverse senescent phenotypes of old HDFs, which are developed spontaneously by a series of cell passage rather than by any exogenous insult, to young-like phenotypes. For this purpose, we first determined optimal concentrations of FAS inhibitors (data not shown). Surprisingly, most senescent phenotypes of old HDFs were effectively reversed by repeatedly exposing the cells to a determined concentrations of FAS inhibitors (cerulenin and C75), though the effect on cell granularity (estimated by SSC) was minor (Fig. 8). Higher concentrations of FAS inhibitors were toxic to cells and single treatment of the low dose was not enough to display the inhibitory effects (data not shown). These data imply that lipogenesis is importantly involved in cellular senescence and pharmacologically blocking of lipogenesis at fatty acid synthesis is an effective approach for attenuating cellular senescence.

DISCUSSION

Increases in cell mass during senescence and the aging process have often been documented (21). Although the detailed components of the mass has not been clearly understood, increasing numbers of reports on the mass of lysosomes and mitochondria in senescence indicate that particular organelles seems to be associated with the mass of the senescent cell (4, 5, 7-9, 21). However, in the present study, we emphasize that, rather than certain organelles, all cellular organelles, particularly membranous organelles, increase during the development of cell senescence, as shown in both stress-induced and replicative senescence. These organelar mass increases correspond well with the increase in membrane lipid content and induction of lipogenic enzymes (FAS, ACC, and ACL) and SREBP-1. This finding implies that enhanced lipogenesis may contribute to the overall organelle increase in cell senescence. Therefore, in preference to investigating the changes or importance of individual organelles, we focused here on exploring the involvement of lipogenesis in the development of senescence. Indeed, enhancing lipogenesis by over-expressing mature SREBP-1 was enough to induce senescent phenotypes in both immortalized Chang cells and primary HDFs. Furthermore, blocking lipogenesis using siRNA-mediated knockdown of SREBP-1 or ACL significantly attenuated H₂O₂-induced senescence. More interestingly, the inhibition of
lipogenesis by FAS-specific inhibitors reversed the senescent phenotypes, not only of the stress-induced senescence, but also of replicative senescence. All of these results demonstrate that enhanced lipogenesis is critically involved in the senescence process, possibly contributing to the membranous organellar mass and, consequently, cell mass (Fig. 9).

The relationship between phospholipid synthesis and senescence or aging has been discussed in only a few reports, though reports on storage fat or oxidatively damaged lipids with aging are increasing (31-33). Inhibitors of HMG-CoA reductase, one of the targets of SREBP, reduce cellular senescence (34, 35), supporting our hypothesis. However, contradictory results have also been reported. Maeda et al. (36) observed that de novo phospholipid synthesis declines in senescent human fibroblasts, though the formation of cholesterol was increased compared to young cells. In that same study, senescent cells developed only after the 25th passage of cell culture, whereas the young HDFs obtained from a 4-year-old boy in our study reached senescence after 75 doublings, implying that the reported cell condition was somewhat different from ours. We also observed that cells in the later stage of senescence (just before reaching cell death) contain much lower levels of lipogenic activity than well-developed senescent cells, with maximum SA-β-gal activity in both stress-induced and replicative senescence (data not shown). Interestingly, increased expression of lipogenesis-associated proteins was further found in the aging F344 rats, presenting the potential involvement of lipogenesis in the aging process. All of these reports and findings further support our hypothesis of the important contribution of enhanced lipogenesis to senescence and aging.

All mammalian cells require the biosynthesis of membrane lipids for the duplication of membranous organelles undergoing cell division. Therefore, further activation of lipogenesis is expected in cancer development, in which the strength of cell growth and proliferation is increased. Indeed, an increasing number of studies on the importance of lipogenesis in carcinogenesis have been reported, and lipid synthesis is regarded as a new target for the development of cancer therapeutics (37-39). However, in terms of cell proliferation, cancer development is the opposite situation to senescence. Therefore, the question of why enhanced lipogenesis leads cells to senescence and not to cell proliferation is important. When we over-expressed mature SREBP-1 protein, we clearly observed p21 and p16 proteins, the negative cell cycle regulators, in addition to the increased expression of lipogenic enzymes. Although protein induction of ACC, the rate-limiting enzyme of fatty acid synthesis, by SREBP-1 was minor, overall flow of fatty acid synthesis may be enhanced by modulation of substrate (acetyl-CoA) and product (malonyl-CoA) levels via the increased ACC and FAS proteins. In addition, the result of the inhibitory cell cycle regulators corresponds well to the previous report that mature SREBP-1 causes cell-cycle arrest through the induction of the cdk inhibitors, such as p21, p27, and p16 (40, 41). Therefore, enhanced lipogenesis via SREBP-1 activation alone may act as an inducer of senescence. In other words, cancer cells should acquire any additional strategy to suppress the action of the cdk inhibitors that may be induced by SREBP-1 in order to use the lipogenic power for cell proliferation.

The question then is why the senescent cells need an increased organellar mass, and whether the organelles are functional. The simple explanations previously applied to lysosomes and mitochondria are possibly applicable to these questions. Senescent cells regenerate organelles in order to compensate for the non-functional organelles damaged by senescence-associated ROS as a defensive response, but the organelles that are supposed to remove the damaged organelles (components), such as lysosomes and proteasomes, have also lost their function, resulting in an accumulation of non-functional organelles (14, 42, 43). The worse scenario is that even newly synthesized organelles may be non-functional due to an oxidatively stressed cellular environment, aggravating or maintaining senescence. However, in question is what happens to the newly generated organelles in the senescence induced by over-expressed mSREBP-1 protein alone, in which no additional stress exist. So far, whether the organelles have normal function, structure, and well-balanced composition,
including membrane lipids and proteins, is not clear. Interestingly, knocking down SREBP-1 reduced the senescent intracellular ROS level, implying that lipogenesis is probably linked to oxidative stress. Therefore, we can hypothesize that enhancement of lipid biosynthesis alone, without supporting other components (for example, proteins) for organelles, may act as a stress by forming only lipid-enriched non-functional organelles. For example, in lipid-enriched mitochondria, respiratory complexes may be dispersed, making their electron transfer reaction defective and increasing ROS generation. However, a detailed understanding of the functional and compositional status of individual organelles in senescence remains unexplored.

SREBP-1 is known to regulate the supply of membrane lipids in response to cell growth and is highly expressed in cells that are actively growing (44). However, SREBP-1-expressing livers of transgenic mice exhibit impaired regeneration after partial hepatectomy (41), indirectly supporting our results that mSREBP-1 over-expression sufficiently induces senescence-associated growth arrest. We also showed that knocking down the up-regulated SREBP-1 in the stress-induced senescent cells effectively attenuated the senescence phenotypes, indicating the potential role of SREBP-1 as a novel effective target for modulating senescence. However, we also observed that knocking down SREBP-1 below the level of control cells by using high dose siRNA also induced growth arrest (data not shown), suggesting that the well-balanced regulation of lipogenesis is required to maintain normal cellular function and the fine-control of SREBP-1 activation is essential to control senescence. In addition, our previous study proved that GSK3 is commonly inactivated by being phosphorylated and is also importantly involved in all the cell senescence systems (DFO- and H2O2-induced, and replicative senescence) employed in this study (27). GSK3 is known to modulate SREBP1 activity through phosphorylation-mediated ubiquitination (45). Therefore, it is suggested that GSK3 may play a critical role in activating SREBP-1 as an upstream modulator in senescence induced by stresses. Detailed link between GSK3 and SREBP1 in senescence is under investigation. Collectively, our results provide new insight into the metabolic background of cellular senescence and indicate SREBP-1-induced lipogenesis as a potentially effective novel target for senescence and aging-related diseases.

REFERENCES


FOOTNOTES
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**Abbreviations used are:** SREBP-1, sterol regulatory-element binding protein 1; ACL, ATP citrate lyase; ACC, Acetyl-CoA carboxylase; FAS, fatty acid synthase, FSC, forward scatter; SSC, side scatter; CP, cholesteryl palmitate; PA, palmitate; CL, cholesterol; CA, cardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; TLC, thin layer chromatography; DFO, deferoxamine; ER, endoplasmic reticulum; ROS, reactive oxygen species; HDF, human diploid fibroblast; SA-β-gal, senescence associated β-galactosidase

**Keywords:** cellular organelles, lipogenesis, metabolism, SREBP-1, transcription factor

**Running title:** Enhanced lipogenesis via SREBP-1 in cell senescence

**FIGURE LEGENDS**

**Fig. 1.** Overall increases of membranous subcellular organelles in cell senescence. A-C. Chang cells were treated with 200 µM DFO or H₂O₂ for 3 days. A. Subcellular organellar masses were estimated by comparing the fluorescence intensities with flow cytometry after staining cells with organelle-specific dyes as described in Experimental procedures. B. (a) Nuclear membrane mass of a cell was represented by single-sectioned area (µm²) of nucleus (nuclei) of each cell. Upper panels are the representative images for both single giant nucleus (SN) and multi-nuclei (MN) of H₂O₂-treated single senescent cell. Ruler bar indicates 5 µM. C. Cellular cytoplasmic membrane mass was represented by single-sectioned area (µm²) of each cell. Upper panels are representative cell phase images of H₂O₂-treated senescent cell. Ruler bar indicates 10 µM. D. Subcellular organellar masses of young (Y, PD 16 or 19) and old (O, PD 79 or 80) HDFs. E. Nuclear membrane mass (a) and cytoplasmic membrane mass (b) of young (Y, PD 16), mid-old (MO, PD 65) and old (O, PD 83) HDFs were also estimated as described in B. **p < 0.01.

**Fig. 2.** Increased lipogenesis in DFO-induced senescence. Chang cells were treated with 200 µM DFO for the indicated periods. A. Cellular lipid profile of senescent cells was obtained by TLC as described in Experimental procedures and figure 2A. Standard lipid mixture (Std) containing 10 µg each was used. Cholesteryl palmitate (CP, 1) belongs to non-polar storage lipid, and cholesterol (CL, 2), cardiolipin (CA, 3), phosphatidylcholine (PC, 4) and phosphatidylserine (PS, 5) belong to membrane lipids. ‘d’ in the x-axis stands for day. B. Western blot for SREBP1. Full length and mature form of SREBP1 were indicated by arrows. C. (a) Western blot analyses for lipogenic enzymes. Expression ratios of ACC (■), ACL (▲), and FAS (◆) to tubulin (b) or lamin (c) are shown.

**Fig. 3.** Increased lipogenesis in old HDFs. A. Cellular lipid profiles of young (PD 15, DT 2) and old (PD 82; DT 9) HDFs were compared by TLC as described in Experimental procedures and figure 2A. Quantitative estimation of TLC analysis of cellular lipids shown in supplementary figure S3 is analyzed. Numbers in the graph stands for the lipid spots on TLC as explained in fig. 2A. B-C. Young (PD 15, DT 2) and old (PD 82; DT 9) HDFs were cultured and applied to Western blot analysis for SREBP-1 (B) and lipogenic enzymes (C, a). Quantitative comparison of the expression levels of the lipogenic enzymes shown in (C, b). *p < 0.05.

**Fig. 4.** Increased lipogenic enzymes and SREBP1 in aging F344 rats. Liver tissues of the aging process of F344 rats (3 individuals for each age) were applied to Western blot analysis. A. Western blot analysis for lipogenic enzymes and SREBP1. B. Expression levels are compared. **p < 0.01 vs. 6 month old rat livers.
Fig. 5. Ectopic expression of mature SREBP-1 induces senescence of Chang cells. Chang cells were infected with recombinant adenovirus encoding mature form of SREBP-1 (ad-mSREBP1) for 4 days. Recombinant adenovirus encoding GFP (ad-GFP) was used as control. A. Western blot analysis of lipogenesis and senescence-related proteins (a). Quantitative analysis of the expression levels of lipogenic enzymes (b). Recombinant mSREBP1 migrates faster than mature form of endogenous human SREBP-1. B. SA-β-gal positive cell population was analyzed (a) and cell growth rates were compared by counting trypan blue-negative live cells (b). Dead cells were hardly observed. C. Cell size (a) and cell granularity (b) were analyzed by comparing FSC and SSC, respectively. R1, R2, R3 and R4 in Y-axis indicates the same window of the cell distribution by flow cytometry as designated in the left panel of Fig. 6B. *p < 0.05, **p < 0.01 vs. ad-GFP.

Fig. 6. Ectopic expression of mature SREBP-1 induces senescence of young HDFs. HDFs (PD 24, DT 2) were infected with ad-GFP or ad-mSREBP1 for 4 days. A. SA-β-gal activities were monitored. Representative images (left panel) and SA-β-gal positive cell populations (right panel) are shown. B. Flow cytometric analysis of the cell distribution. Representative profiles (left) and cell granularity (SSC analysis, right) are shown. C. Cell growth rates. D. Western blot analysis (a) and quantitative analysis (b) of lipogenic enzyme expressions are shown.

Fig. 7. SREBP-1 knockdown attenuates H2O2-induced senescence of Chang cells. A-C. Chang cells (1 X 10^4) seeded on 6 well plates were transfected with siRNAs for SREBP-1 (si-SRE) and then treated with 200 μM H2O2 for 4 days. A. (a) Knockdown of SREBP-1 expression was confirmed by Western blotting. (b) SA-β-gal activities. B. Cell size and cell granularity were estimated by comparing FSC (a) and SSC (b) of cell distribution with flow cytometry, respectively. (c) Cell growth rate was compared by counting trypan blue-negative live cell. Dead cells were hardly observed. (d) Intracellular ROS levels were estimated by flow cytometric analysis of DCF fluorescence after staining cells with DCFH-DA. C. Chang cells (1 X 10^4) were treated with siRNAs for ACL and then treated with 200 μM H2O2 for 4 days. SA-β-gal activities (a) and Western blot analysis of ACL (b) were shown. D. Chang cells were pre-treated with the indicated concentrations of cerulenin (Cer, Sigma C2389) or C75 (Sigma, C5490) and then treated with 200 μM H2O2 for 4 days. SA-β-gal activities were performed. Two different siRNAs for each protein (SREBP-1 or ACL) were used. siRNA of random sequence was used as negative control (NC) and DMSO was used as vehicle (V). ***p < 0.01 vs. no H2O2. **p < 0.01 vs. NC or V.

Fig. 8. Reversal of senescent phenotypes of old HDFs by pharmacological inhibition of lipogenesis. Old HDFs (PD 73, DT 8) were treated with the indicated concentrations of FAS inhibitors and replenished with new medium containing the inhibitors every other day for two weeks. A. Cell growth rate was estimated by counting trypan blue negative cells. Dead cells were hardly detected. B. SA-β-gal activity. C. Cell size was estimated by FSC analysis with flow cytometry. D. Cell granularity was estimated by SSC analysis with flow cytometry. *p < 0.05, **p < 0.01 vs. vehicle.

Fig. 9. Schematic diagram for involvement of lipid biosynthesis in cellular senescence.
Figure 1. Increase of cellular organelles in stress-induced senescence

A

MitoTracker  
LysoTracker  
ER Tracker  
BODIPY

Fluorescence  (Arbitrary unit, mean)

C  DFO  H2O2

B

Con  H2O2 (SN)  H2O2 (MN)

Nucleus area (μm²)

Con  DFO  H2O2

C

Con  H2O2

Cell area (μm²)

Con  DFO  H2O2

D

MitoTracker  
LysoTracker  
ER Tracker  
BODIPY

Fluorescence  (Arbitrary unit, mean)

Y  O  Y  O  Y  O  Y  O

PD:  19  79  PD:  16  79  PD:  16  80  PD:  16  79
E (a) Nucleus area (µm²)

(b) Cell area (µm²)

PD: 16 65 83

Nucleus area (µm²)

Cell area (µm²)
Figure 2. Increased lipogenesis in DFO-induced senescence

A (a)  

B  

C (a)  

(b)  

Expression ratio (Enzymes/tubulin)  

Expression ratio (Enzymes/lamin)
Figure 3. Increased lipogenesis in replicative senescence

A

Cellular lipids (% control)

HDF: Young  Old
PD: 15  82

B

Full length

Mature form

Tubulin

SREBP1

PD 13  15  76  81 (d)

HDF: Young  Old

C (a)

FAS

ACC

ACL

Tubulin

PD 13  15  76  81 (d)

DT 1.5  1.5  14  16 (d)

HDF: Young  Old

(b)

Expression

Y O Y O Y O

FAS  ACC  ACL

*
Figure 4. Increased expression of lipogenic enzymes and SREBP-1 in aging F344 rats.
Figure 5. Ectopic expression of mature SREBP-1 induces senescence of immortalized Chang cells

A (a) 

B (a)

C (a)

Expression Ratio (ad-mSREBP1/GFP)

FSC increase (Cells in R2+R4, %)

SSC increase (Cells in R1+R2, %)

** ** **

Figure 5. Ectopic expression of mature SREBP-1 induces senescence of immortalized Chang cells

A (a) 

B (a)

C (a)

Expression Ratio (ad-mSREBP1/GFP)

FSC increase (Cells in R2+R4, %)

SSC increase (Cells in R1+R2, %)

** ** **

Figure 5. Ectopic expression of mature SREBP-1 induces senescence of immortalized Chang cells

A (a) 

B (a)

C (a)

Expression Ratio (ad-mSREBP1/GFP)

FSC increase (Cells in R2+R4, %)

SSC increase (Cells in R1+R2, %)

** ** **

Figure 5. Ectopic expression of mature SREBP-1 induces senescence of immortalized Chang cells

A (a) 

B (a)

C (a)

Expression Ratio (ad-mSREBP1/GFP)

FSC increase (Cells in R2+R4, %)

SSC increase (Cells in R1+R2, %)

** ** **
Figure 6. Ectopic expression of mature SREBP-1 induces senescence of primary young HDFs

A

ad-GFP
ad-mSREBP1

B

Side Scatter

Forward Scatter

C

Cell Number (X10^4 cells)

ad-GFP   ad-mSREBP1

D (a)

Full length
Mature form
mSREBP1

SREBP1
SREBP2

D (b)

Expression Ratio (ad-mSREBP1/GFP)

0 20 40 60 80

FAS
ACC
ACL

100 25 50 100 (MOI)

ad-GFP   ad-mSREBP1

100 25 50 100 (MOI)

GFP   ad-mSREBP1
Figure 7. Blocking lipogenesis attenuates H$_2$O$_2$-induced senescence

A (a) SA-ß-gal (+) cells (% total cell)

B (a) FSC increase (Cells in R2+R4, %)

C Cell Number (X10$^4$ cells)

D DCF Fluorescence (Arbitrary U, mean)
Figure 7. – continue

C (a)

D (a)

siRNA:

100   100   30   100    30    100
(pmole) ACL(#1) (#2)NC

H2O2:

- +      +     +     +      +

Inhibitor:   - - 30    100   100   300
(nM) V Cer C75

H2O2:     - +      +     +     +      +

Cer C75V

SA-β-gal (+) cells (% total cell)

SA-β-gal (+) cells (% total cell)

(a) (b) (a) (b)

ACL p21
Tubulin

ACL p21
Tubulin

H2O2:

- +      +     +     +      +

- +      +     +     +      +

NC ACL(#1) (#2)

NC ACL(#1) (#2)
Figure 8. Reversal of senescence of old HDF by lipogenesis inhibition
**Figure 9. Model of Lipogenesis Enhancement in Cellular Senescence**

Stress

\[ \text{mature SREBP-1} \uparrow \]

\[ \text{TCA} \]

\[ \text{Citrate} \rightarrow \text{ACL} \rightarrow \text{Acetyl CoA} \]

\[ \text{ACC} \rightarrow \text{Malonyl-CoA} \rightarrow \text{FAS} \rightarrow \text{Fatty Acids} \]

\[ \text{Lipogenesis Enhancement} \]

\[ \text{Membranous organelle mass increase} \]

A novel Senescence Indicator

Typical Senescence Phenotype: Cell mass increase

Cellular Senescence
Sterol regulatory element binding protein (SREBP)-1-mediated lipogenesis is involved in cell senescence
You-Mie Kim, Hyun-Taek Shin, Yong-Hak Seo, Hae-Ok Byun, Soo-Han Yoon, In-Kyu Lee, Dong-Hoon Hyun, Hae-Young Chung and Gyesoon Yoon

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