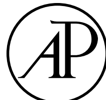
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Biochemical and Biophysical Research Communications 295 (2002) 187–192

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# Effects of histone acetylation on transcriptional regulation of manganese superoxide dismutase gene

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Received 6 June 2002

**Abstract**

To better understand the link between chromatin modification and manganese superoxide dismutase (Mn-SOD) gene expression, we have investigated the level of histone acetylation at Mn-SOD proximal promoter. TSA induced the expression of Mn-SOD mRNA and its transcriptional activity in C2C12 cells. Sp1 binding sites in the proximal promoter region of Mn-SOD were transcriptionally responsive to TSA by transfection studies. We have detected a localized acetylation of histones H3 and H4, in vivo occupation by Sp1, early growth responsive-1 (Egr-1), and histone deacetylase-1 (HDAC1) in the proximal promoter region of Mn-SOD gene using chromatin immunoprecipitation assays. Our findings indicate that Mn-SOD gene expression is repressed by Sp1–HDAC1 complex. This repression is released by a localized histone acetylation and at least in parts a displacement by Egr-1 in response to TSA. © 2002 Elsevier Science (USA). All rights reserved.

*Keywords:* Manganese superoxide dismutase; Histone acetylation; Transcription; Histone deacetylase-1; Transcription; Sp1; Egr-1

The acetylation of histones is thought to be involved in the destabilization and restructuring of nucleosomes, which is probably a crucial event in the control of the accessibility of DNA templates to transcriptional factors [1–3]. A current working hypothesis is that recruitment of coactivators with HAT activity by promoter-bound transcription factors results in the acetylation of histone residues of nearby nucleosomes, which increases the accessibility of the DNA to the transcription machinery [4–6]. Replicative senescence has been studied using in vitro cultured cells (reviewed in [7–9]). This system has provided information relevant to in vivo aging processes. Senescent cells show a number of phenotypic traits. The cells become enlarged with a flattened and irregular shape, exhibit increased senescence-associated β-galactosidase activity (SA-β-gal), and up-regulate the cyclin-dependent kinase inhibitors (CKIs), such as $p21^{Cip1/Waf1}$ (p21) and $p16^{INK4a}$ (p16). A wide spectrum of signals, DNA damage, oxidative stress, histone deacetylase (HDAC) inhibitors [10,11], and expression of activated oncogenes, such as ras [12], raf [13], or mitogen-activated protein kinase kinase (MEK) [14], can cause phenotypes closely resembling senescence, referred to as premature senescence. Inhibitors of HDACs, such as sodium butyrate and trichostatin A (TSA), can induce premature senescence in human diploid fibroblasts [10] and the expression of p21 in some cultured cells [11,15]. There is a significant decrease in the level of HDAC1 during replicative senescence in human fibroblasts [16]. In addition to the evidence from in vitro cultured system, silent information regulator 2 (Sir 2), a nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylase, is a limiting component that promotes longevity in yeast [17,18]. A line of evidence indicates that HDACs involve in controlling the expression of subsets of genes related to senescence.

To examine the involvement of histone acetylation in Mn-SOD gene expression, we have investigated the acetylation level of histones at Mn-SOD proximal promoter and related factors in response to TSA, an

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PII: S0006-291X(02)00646-0

inhibitor of HDACs. Our findings showed that Sp1–HDAC1 complex repressed Mn-SOD gene expression in untreated condition. The epigenetic regulation of Mn-SOD by Sp1–HDAC1 complex may contribute to enhance resistance to oxidative stress in senescence. This repression was released in TSA-treated cells by two distinct mechanisms, a rapid acetylation of histones H3 and H4 and a displacement of Sp1–HDAC1 complex by early growth responsive-1 (Egr-1) induced by TSA.

## Materials and methods

*Materials.* C2C12 cells were obtained from RIKEN CELL BANK. Cell culture reagents were obtained from Gibco-BRL Life Technologies. Fetal bovine serum and TSA were obtained from Sigma. Antibodies were from the following sources: anti-acetylated histone H3, anti-acetylated histone H4, anti-Sp1, and anti-HDAC1 from Upstate Biotechnology; anti-Egr-1 and control rabbit immunoglobulin G (IgG) from Santa Cruz Biotechnology; horseradish peroxidase (HRP)-conjugated anti-rabbit-IgG from New England Biolabs. An Sp1 consensus oligonucleotide was obtained from Santa Cruz Biotechnology.

*RNA isolation and analysis.* The cells were preincubated in fresh serum-free medium for 24 h before the treatments. Total cytoplasmic RNA was isolated using the guanidine isothiocyanate method from the cells that were left untreated or treated with 100 ng/ml TSA for 2, 4, 8, 16, or 24 h. Samples of 10 μg total RNA were denatured, separated by electrophoresis in a 1% agarose gel containing formaldehyde, and transferred to GeneScreen membranes (DuPont, NEN). The membranes were prehybridized and then hybridized with a Mn-SOD cDNA [19], Sp1 cDNA [20], or Egr-1 cDNA probe [21] labeled with [$\alpha$-$^{32}$P] dCTP (NEN) using a random primer labeling system (Pharmacia Biotech). After hybridization, the membranes were washed and exposed to X-ray film (Fuji Film). All blots were rehybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe to normalize for mRNA loading differences. To quantify the contents of mRNA in the cells, the membranes were exposed to imaging plates (Fuji Film) and radioactivities were measured with a bioimage analyzer, Fijix BAS 1500 (Fuji Film).

*Cell culture.* C2C12 cells were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin.

*Plasmid constructs.* The mouse Mn-SOD promoter luciferase reporter plasmids were constructed as described previously [21]. The construction of reporter plasmids containing mutated sequences in Sp1 binding sites was described previously [22].

*Transfection assays.* Transient transfection of C2C12 cells was carried out using SuperFect Reagent (Qiagen) as previously described [22]. In general, the day before transfection, $2 \times 10^5$ cells were plated in 12-well tissue culture plates. A total of 2.5 μg DNA consisting of 2.25 μg indicated luciferase plasmid and 0.25 μg pRL-thymidine kinase control vector (pRL-TK) (Promega) per plate was used for transfection studies. After transfection, the cells were placed in serum-free medium for 24 h; 100 ng/ml TSA or control buffer was then added for an additional 16 h. After being harvested, the cells were assayed by the Dual-Luciferase Reporter Assay System (Promega), using a luminometer (EG & G Berthold). Protein concentrations of the cell lysates were determined by the method of Bradford with the Bio-Rad Protein Assay Dye Reagent. Promoter activities were expressed as relative light units (RLU), normalized against the concentration of the protein. For transfection assay using reporter plasmids containing mutated sequences in Sp1 binding sites, the luciferase activity of Mnpro (−250) in untreated cells was normalized to 100 and the relative activities were shown. All transfection experiments were repeated three times.

*Western blot analysis.* Nuclear extracts (20 μg) were run under reducing conditions in 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA), and reacted with antibodies specific for Sp1 and Egr-1. The primary antibodies were detected by counterstaining with a HRP-linked antibody and visualized by enhanced chemiluminescence.

*Chromatin template immunoprecipitation (Chip).* Soluble chromatin was prepared according to manufacturer's recommendations with slight modification. The equivalent of $1 \times 10^6$ cells was used per chromatin immunoprecipitation reaction. Approximately $6 \times 10^6$ C2C12 cells were grown and preincubated in fresh serum-free medium for 24 h before the treatments. Cells treated with or without 100 ng/ml TSA for indicated time were fixed by adding formaldehyde to 1% final concentration. Cross-linking was allowed to proceed at room temperature for 6 min and terminated with glycine (final concentration 125 mM). Cells were washed with PBS, collected in lysis buffer, and sonicated on ice. A mean DNA size generated by sonication was 0.3–1.0 kb. Chromatin was precleared with salmon-sperm DNA/protein An agarose slurry for 1 h at 4 °C with rotation. Precleared chromatin was incubated with 2.5 μg of each antibody specific for acetylated histones H3 and H4, Sp1, and HDAC1 or 4 μg anti-Egr-1 antibody at 4 °C with rotation. Immunocomplexes were recovered by adding 30 μl salmon-sperm DNA/protein A–agarose slurry for 1 h at 4 °C with rotation. Precipitates were successively washed with following buffers: low salt immunocomplex wash buffer (0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl (pH 8.1), and 150 mM NaCl), high salt immunocomplex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl (pH 8.1), and 500 mM NaCl), LiCl immunocomplex wash buffer (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, and 10 mM Tris–HCl (pH 8.1)), and 1 × TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) twice. Pellets were eluted two times in 250 μl elution buffer (1% SDS, 0.1 M $NaHCO_3$). Cross-links were reversed by incubating samples at 65 °C overnight. Samples were incubated with 20 μg proteinase K at 45 °C for 1 h, extracted with phenol:chloroform, and ethanol precipitated. Pellets were resuspended in 50 μl $H_2O$ and utilized for quantitative polymerase chain reaction (PCR) analysis. PCR were performed in 25 μl volume with immunoprecipitated material, 10 pmol primer set, 0.5 U KOD plus DNA polymerase (Toyobo), and 1 μCi [$\alpha$-$^{32}$P]dCTP. After 2 min of denaturation at 94 °C, samples were subjected to 30 cycles of a program consisting of 15 s at 94 °C and 1 min at 68 °C, ending with a final 7 min incubation at 72 °C. The linear range for the primer pair was determined using different amounts of input samples. The primers used to amplify Mn-SOD promoter region were 5′-GCTCGCTGG TCCGGGATGGCAGCGGCC-3′ (positions −263 to −237) and 5′-CTTTTATTCCCTTTAGCAAATTTCAA-3′ (positions −107 to −89). PCR products were electrophoresed on 8% polyacrylamide gels and quantified on a Fuji phosphorimager.

## Results and discussion

### *TSA enhances the expression of Mn-SOD*

C2C12 cells were treated with TSA to examine the effect on Mn-SOD gene expression. Mn-SOD mRNA was increased in the first 2 h with TSA treatment. The level of Mn-SOD mRNA in TSA-treated cells peaked at 8 h with a threefold induction compared to the level in the untreated cells (data not shown).

*Mn-SOD promoter contains regulatory DNA elements responsive to TSA*

A series of Mn-SOD promoter plasmids was transiently transfected into C2C12 cells and then treated with 100 ng/ml TSA for 16 h. The promoter activity was normalized against the concentration of the protein, because the activity of pRL-TK control vector was induced by TSA. Constructs containing 231, 250, 538, and 1452 bp of 5′ flanking DNA exhibited a high

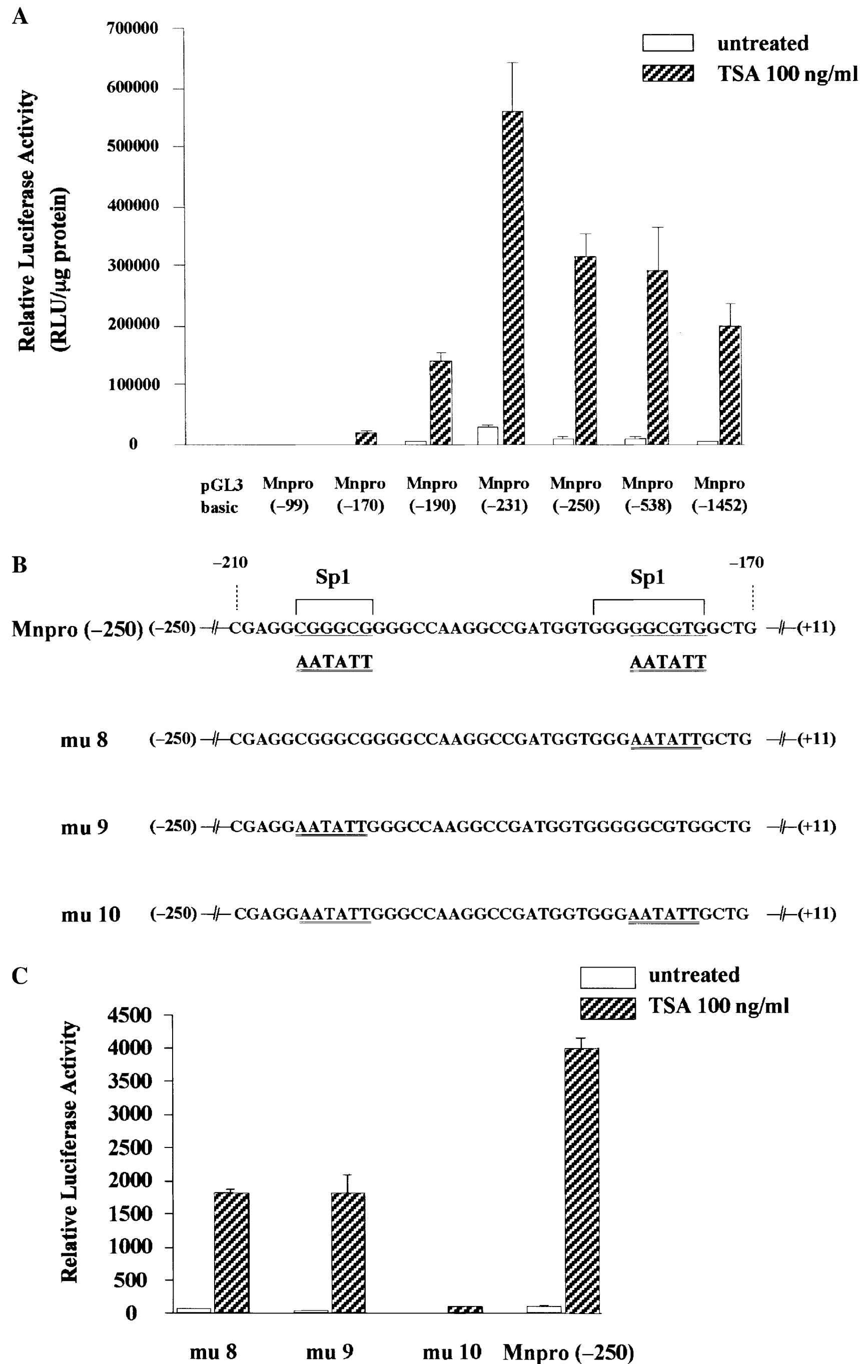


Fig. 1. Functional analysis of the Mn-SOD promoter. (A) C2C12 cells were transfected with luciferase reporter constructs containing the 5′ flanking DNA of the Mn-SOD gene and then treated with 100 ng/ml TSA for 16 h. Promoter activities are expressed as relative light units (RLU), normalized against the concentration of the protein. The positions of the bases are indicated relative to the Mn-SOD transcription initiation site (+1). (B) Reporter plasmids containing mutated sequences (mu 8, mu 9, and mu 10) in Sp1 binding sites were used for transfection studies. Brackets indicated the location of the sites for Sp1. (C) C2C12 cells were transfected with luciferase reporter constructs containing mutated sequences and then treated with 100 ng/ml TSA for 16 h. The luciferase activity of Mnpro (−250) in untreated cells was normalized to 100 and the relative activities were shown. Results are expressed as means ± SE of three independent experiments, each performed in triplicate.

level of relative luciferase activity in the untreated cells and the activity was increased drastically by the treatment with TSA (Fig. 1A). A 231-bp fragment of Mn-SOD proximal promoter contained two GC clusters. To clarify the *cis*-elements for TSA-mediated Mn-SOD transcription, we performed luciferase assays with reporter plasmids containing the site-specific mutations introduced into the Mnpro (−250) (Fig. 1B). The mutations of the Sp1 binding sites (Sp1 and Egr-1 binding sites) reduced the transcriptional activity in untreated cells (Fig. 1C). Abrogation of one of the Sp1 sites (mu 8 and mu 9) caused a 50% reduction of TSA-induced transcriptional activity compared with that of the cells transfected with Mnpro (−250). Mutations of both sites for Sp1 (mu 10) led to a marked loss of TSA-induced transcriptional activity. Transfection studies indicated that Sp1 protein was a crucial factor for TSA-mediated Mn-SOD transcription. EMSA experiments showed that the binding elements of Egr-1 and Sp1 family proteins overlapped and Egr-1 competed with Sp1 for binding to Mn-SOD promoter (data not shown).

*Acetylation level of histones H3 and H4 correlates with Mn-SOD transcriptional activation*

We investigated the possibility that TSA stimulation results in the acetylation of histones H3 and H4 within Mn-SOD promoter. We immunoprecipitated formamide cross-linked, sonicated chromatin fragments from the untreated cells and TSA-treated cells using antibodies specific for the acetylated histones H3 and H4 (Figs. 2A and B). Input corresponded to PCR containing 0.5% total amount of chromatin used in immunoprecipitation reactions. An enhancement of acetylated histones H3 and H4 was rapidly observed in Mn-SOD proximal promoter after TSA stimulation in C2C12 cells. Increased acetylation level of histones H3 and H4 sustained over twofold in 6 h after stimulation compared with the level of untreated cells. The levels of localized acetylation of histones H3 and H4 declined gradually and reduced to the level of the untreated cells in 15 h and 24 h after stimulation, respectively. A localized acetylation of histones H3 and H4 at Mn-SOD promoter occurred concomitantly with its gene expression in TSA-treated cells (Fig. 2B).

*In vivo occupancy by Sp1, Egr-1 and HDAC1*

Mn-SOD promoter has GC clusters bound by Sp1 family proteins and Egr-1 (data not shown). We performed Chip assays to analyze in vivo occupancy of the related factors to Mn-SOD promoter (Fig. 2). Sp1 binding to the proximal promoter was detected in the untreated cells. The binding by Sp1 was increased in 1 h and then reduced by 9 h, following TSA treatment. After

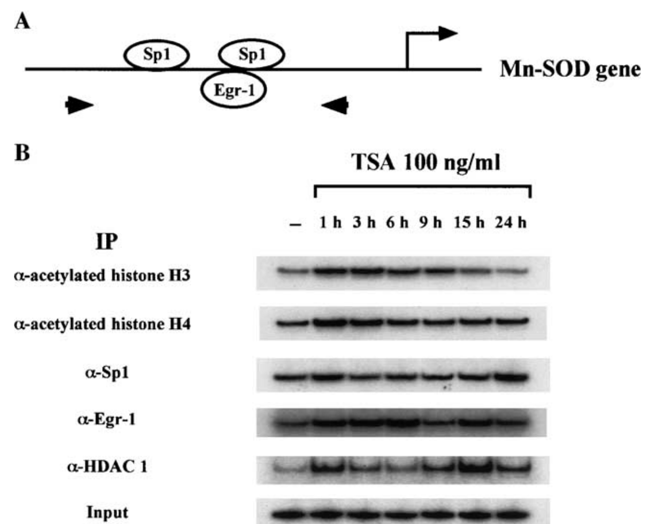


Fig. 2. A localized histone acetylation and in vivo occupancy by Sp1, Egr-1, and HDAC1 in Mn-SOD promoter. (A) Mn-SOD proximal promoter was examined in Chip assay. Sp1 and Egr-1 binding sites are shown. Small arrows indicated a primer pair of Mn-SOD gene, positions −263 to −237 and −107 to −89. Large arrow indicated a transcription start site. (B) Antibodies specific for acetylated histones H3 and H4, Sp1, Egr-1, and HDAC1 were used to immunoprecipitate chromatin. PCR was performed with the primer pair of Mn-SOD gene. Amplified products were detected by autoradiography. Input corresponded to PCR containing 0.5% total amount of chromatin used in immunoprecipitation reactions.

that, Sp1 occupancy was gradually increased again in TSA-treated cells. In contrast to Sp1 binding, the intensity of Egr-1 binding to Mn-SOD promoter gradually increased from 1 h to 3 h and peaked at 6 h in TSA-treated cells. This result was consistent with the results that Egr-1 was synthesized in response to TSA shown in Figs. 3A and B. In vivo occupancy by HDAC1 to the promoter was slightly detected in the untreated cells and strongly detected in the cells within 1 h after TSA stimulation. HDAC1 occupancy was drastically reduced in the treated cells for 6 h and then increased by 15 h after TSA treatment. This pattern was reminiscent of that of Sp1 binding observed in the cells, indicating the possibility that HDAC1 was recruited by the association with Sp1.

*Egr-1 expression is induced by TSA*

We analyzed the expression of Sp1 and Egr-1 in response to TSA. Nuclear extracts were subjected to SDS–PAGE and Western blot analysis. As shown in Fig. 3A, Sp1 family proteins were expressed constitutively in nuclear extracts prepared from both untreated and TSA-treated cells. In contrast, Egr-1 was induced in the nuclear extracts prepared from TSA-treated cells. The expression of Sp1 mRNA was not altered by TSA treatment (Fig. 3B). After TSA stimulation, the induc-

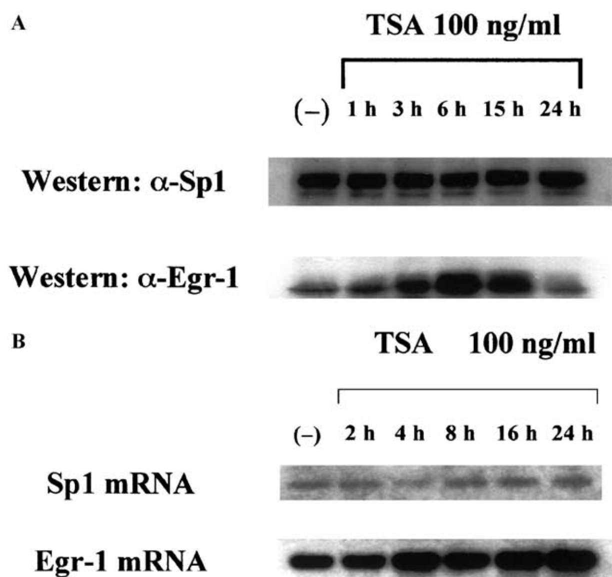


Fig. 3. TSA induces Egr-1 in C2C12 cells. C2C12 cells were left untreated or treated with 100 ng/ml TSA for the indicated time. (A) Nuclear extracts were subjected to Western blot analysis using anti-Egr-1 or Sp1 antibody. (B) Northern blot analysis using an Egr-1 or Sp1 cDNA probe.

tion of Egr-1 mRNA was detected within 4 h and maintained by 24 h.

Transcriptional activation of Mn-SOD induced by TSA was mediated by two distinct mechanisms. One is an inhibitory effect of TSA on HDAC1 activity essential for regulation of transcription. Inhibition of HDAC1 activity resulted in a rapid acetylation of histones H3 and H4 around the proximal promoter region of Mn-SOD transcriptionally responsible for TSA. We could not exclude the contribution of the other HDACs to Mn-SOD transcription. Another is a displacement of Sp1–HDAC1 complex by Egr-1 in response to TSA. Egr-1 was found to compete with Sp1 in the binding to Mn-SOD promoter shown in EMSAs and Chip assay. We previously showed that platelet-derived growth factor (PDGF) induced Mn-SOD gene expression and the mechanism of Mn-SOD transcription mediated by PDGF involved MEK1-extracellular signal-regulated kinase 1 and 2 (ERK1/2) signaling pathway and its downstream transcription factor, Egr-1 [21]. The induction of Egr-1 occurred more rapidly in PDGF-treated cells than in TSA-treated cells. This competition may play a role in regulation of promoter activity of Mn-SOD.

## Acknowledgments

We thank Drs. Ye-Shih Ho (Wayne State University) for providing a rat Mn-SOD cDNA probe and Thomas Shenk (Princeton University) for providing pCGN-Sp1. We greatly thank Dr. Yasuhiko Takahashiwho who enabled us to establish the chromatin immunoprecipitation assay in our own laboratory. We are indebted to Drs. Hideo Nakajima and Mitsuo Maruyama for many helpful comments and suggestions. This study was supported by Fund for Comprehensive Research on Aging and Health, in part by the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Pharmaceutical Safety and Research (of Japan) and in part by grant-in-aids for Scientific Research from the Ministry of Education.

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