Transcriptional Activation of the Cu,Zn-Superoxide Dismutase Gene through the AP2 Site by Ginsenoside Rb2 Extracted from a Medicinal Plant, Panax ginseng

(Received for publication, April 2, 1996, and in revised form, July 11, 1996)

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We report here that the ginseng saponins induce the transcription of Cu,Zn-superoxide dismutase gene (SOD1), which is one of the major antioxidant enzymes. Total saponins and panaxatriol did not elevate the level of SOD1, but panaxadiol significantly increased SOD1. Among the panaxadiol fractions, ginsenoside Rb2 was a more specific and more remarkable inducer of the SOD1 gene than ginsenoside Rb1. Deletion analyses of the SOD1 promoter revealed that the proximal promoter is responsible for this induction. Mobility shift assays with cis-elements in the proximal promoter region showed that specific binding of the AP2 transcription factor was significantly increased by treatment with ginsenoside Rb2. These results suggest that the SOD1 gene was greatly activated by ginsenoside Rb2 through transcription factor AP2 binding sites and its induction.

Cu,Zn-superoxide dismutase (SOD1) is a key enzyme in the metabolism of oxygen free radicals. It catalyzes the dismutation of superoxide radicals (O$_2^-$) to oxygen and hydrogen peroxide (1). Generation and/or removal of superoxides have been observed to play significant roles in a variety of critical homeostatic mechanisms both at the cellular and the organismic level. Since biological macromolecules would be a target for the damaging action of abundant oxygen radicals, the regulation mechanism of the SOD1 gene would be of great interest. It has been also reported that SOD1 could prevent oncogenesis and tumor promotion (2), reduce the cytotoxic and cardiotoxic effects of anticancer drugs (3), and protect against reperfusion damage of ischemic tissue (4). A recent report suggested that overexpression of SOD1 and catalase could increase the average life span of the fly (5).

In yeast, the expression of SOD1 is regulated by copper at the level of transcription (6). Lutropin, Cu$^{2+}$ ion, and reactive oxygen seemed to induce SOD1 in rat (7–9). The rat SOD1 promoter region has recently been studied for possible regulatory elements that could account for its induction by various agents (10). Functional heat shock elements within the human SOD1 promoter have been identified, which suggests that heat shock factors mediate the activation of SOD1 gene transcription under a heat shock stress condition (11). The diversity of SOD1 inducers implies that there are multiple regulatory elements for this gene. Indeed, the present study identified additional regulatory elements, binding sites for AP2, which would be an important factor mediating the role of SOD1 in the response to oxidative stress.

Panax ginseng C. A. Mayer (Araliaceae) is one of the most popular natural tonics that has been used in oriental countries. Ginseng showed antitumor activities in slow growing tumors but not in rapidly growing tumors (12). Ginseng also inhibited tumor angiogenesis and metastasis (13). In an epidemiological study, the intake of ginseng reduced the incidence of human cancer (14). These antitumor effects of ginseng support the suggestion of Brekhman that ginseng may increase the nonspecific resistance of the organism (15). However, the identity of the active substance and its mechanism of action have not been elucidated yet. Ginsenosides can be classified into panaxadiol (PD) and panaxatriol (PT) saponins according to their sugar moiety of the molecule, have been isolated and identified to date (17). In this study, we examined the effect of ginseng saponins on the transcription of the rat SOD1 gene, which is very similar to that of humans. They have an almost identical proximal part of the promoter region (11). We identified the active fraction of ginseng saponin and further characterized ginsenoside Rb2 (Rb2) as a strong transactivator of the SOD1 gene through AP2 binding sites and its induction.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonuclease and DNA-modifying enzymes were obtained from New England Biolabs. The synthetic oligonucleotides for transcription factor binding site were purchased from Promega. [$\gamma$-32P]ATP and [$\alpha$-32P]dATP were from Amersham Corp. All other chemicals were of analytical or molecular grade and were purchased from Sigma.

Vector Constructions—The 1.7-kilobase pair BamHI/SmaI fragment (nucleotides −1633 to +85) from the rat SOD1 gene (10) was inserted into the pBLCAT3 (18). Unidirectional 5′ deletion mutants were produced by cutting 5′ of the SOD1 promoter with SpiI and BamHI, followed by subsequent treatment with exonuclease III (19). Deletion end points were confirmed by DNA sequencing with the Sequenase kit (U. S. Biochemical Corp.). For the construction of pAP2ω, the oligonucleotide of the AP2 binding sequence, which corresponded to the SV40 AP2 binding site, was cloned into the BamHI site of pBLCAT2Δ, which is derived from pBLCAT2 (18). The plasmid pBLCAT2Δ has the mini-
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Cell Culture, Transfection, and Treatment of Ginseng Saponins and Chemicals—Human HepG2 hepatoma cells were grown in Dulbecco’s modified Eagle’s medium, 10% fetal calf serum, penicillin G sodium (100 U/ml), streptomycin sulfate (100 µg/ml), and amphotericin B (250 ng/ml). Cells were seeded into 60-mm plastic culture dishes (30–50% confluence) for 24 h prior to transfection. An equal amount (3.0 pmol) of the various constructs was transfected to the cells by the calcium phosphate DNA coprecipitation method (20). Five µg of pRSVjgal plasmid (21) was introduced in all experiments to correct the variations of transfection efficiency. Ginseng saponins were added to culture medium at 36 h after transfection, and the cells were maintained for an additional 22 h. To determine the maximum induction time of Rb₂, the growth medium was removed, and the Rb₂ was added to the cells at 50 µM in phosphate-buffered saline (PBS). Treatments were maintained for 30 min at 37°C. After treatments the growth medium was added back to the cells, and incubation was continued for the intervals indicated in Fig. 1. In order to determine the new RNA and protein synthesis required, cultures of HepG2 cells were treated with either actinomycin D (2.5 µg/ml) or cyclohexamide (10 µg/ml), or left untreated. After 1 h, cultures were treated with ginsenoside Rb₂ (50 µM) for 12 h.

β-Galactosidase and CAT Assays—The CAT assay was performed as described previously (22). The transfected cells were washed twice with PBS and harvested. The pelleted cells were resuspended in 100 µl of 0.25 M Tris-Cl (pH 7.6) and lysed by three cycles of freezing and thawing. After removal of cell debris by centrifugation, cell extracts were first assayed for β-galactosidase activity (23). Equal quantities of proteins were assayed for CAT activity on the basis of β-galactosidase activity. Extracts were incubated with 0.025 µCi of [3H]chloramphenicol, 0.25 µl Tris-Cl, pH 7.6, 0.4 µM acetyl coenzyme A for 1 h at 37°C. The enzyme assay was terminated by adding ethyl acetate. The organic layer was analyzed by TLC with chloroform/methanol (95:5). After autoradiography, both acetylated and unacylated forms of [3H]chloramphenicol were scraped from the plate, and the conversion of chloramphenicol to acetylated form was calculated by measuring radioactivities. The relative CAT activities were calculated from the percentage of conversion. Results are the average of three independent experiments.

Mobility Shift Assay (MSA)—The double-stranded oligonucleotides corresponding to the metal binding factor, heat shock factor, Sp1, AP2, CCAAT-enhancer binding protein (C-E-BP), metal binding factor, heat shock factor, Sp1, AP2, and SOD1 promoter. Two copies of the AP2 consensus oligonucleotide were introduced. The plasmid pApA2m is a mutant of pApA2w with two copies of the mutated AP2 site (from CCCTCGGC to CCATATGC). Insertion of the AP2 consensus and mutant sequences was confirmed by DNA sequencing. The plasmid pRS-305ApA2m was constructed as follows: DNA fragment (HincII-NcoI) from pRS-305 (see Fig. 2) was excised and ligated with synthetic oligonucleotide containing the mutated AP2 binding site. The AP2 binding sequences located at –124 and –118 were replaced with mutated sequence at –134, from CCCTCGCC to CCATATGC; at –118, from CCCTCGGC to CCATATGC.

RESULTS

Effect of Ginseng Saponins on the Expression of Rat Cu,Zn-SOD—The effect of ginseng saponins on the induction of the SOD1 gene was examined as follows. At first, pRSVP-1633, which is the SOD1 promoter region-CAT fusion plasmid (Fig. 2B), was introduced into HepG2 cells (18). Thirty-six h after transfection, ginseng saponins were added to the transfected cells to an appropriate concentration (Fig. 1, A–D). After 22 h, the CAT activity of each transfected cell was determined. Total saponins (TS) and PT treatment of the cell had no effect on the transcription of the SOD1 gene, but PD increased SOD1 about 3-fold (Fig. 1B). PD consisted of ginsenoside Rb₁ (Rb₁), Rb₂, and some other minor fractions. Therefore, we tested which subfraction of PD had an effect on the induction of the SOD1 gene. Rb₁ and Rb₂ were tested, and ginsenoside Rg₁ (Rg₁), which is a major subfraction of PT, was also tested as a negative control. Fig. 1C showed that Rg₁ had no effect, as expected, whereas Rb₂ was a 2-fold more potent activator than Rb₁. Treatment of the cell with increased amounts of Rb₂ resulted in a gradual increase in the CAT activity (Fig. 1D). This result confirmed again the Rb₂-specific activation of the SOD1 gene. To characterize the induction profile, transfected cells were treated with 50 µM Rb₂ in PBS for 30 min. After incubation, the cells were washed with PBS, and then fresh growth medium was added. The peak of induction appeared around 2 h after treatment and then declined (Fig. 1E). This phenomenon was similar to the
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**Fig. 2.** Deletion analysis of the upstream region of SOD1 gene for the identification of the target region responsible for the induction by the ginsenoside Rb₂. A, the numbers in the name of plasmids represent the deletion end points. CAT activities with (+) or without (−) Rb₂ were indicated by the solid bars and blank bars, respectively. B, schematic representation of the putative transcription factor binding sites and approximate location in the SOD1 promoter. PPAR, peroxisome-proliferating activator receptor; PRE, positive responsive element; NRE, negative responsive element; MFB, metal binding factor; HSF, heat shock factor; CCAAT, CAT box; TATA, TATA box. The numbers represent the left end point of the consensus sequences of each transcription factor binding site.

induction profile of heme oxygenase by oxidative stress (25). It is possible that transmission of the Rb₂ signal to induce the SOD1 gene could be mediated through the DNA element located in the promoter region.

**Identification of Ginsenoside Response Element in the SOD1 Promoter**—To identify the target sequence of Rb₂ in the upstream region of the SOD1 gene, deletion mutants were prepared and transfected into the HepG2 cell with or without Rb₂ in the medium. As outlined in the bar graph of Fig. 2A, deletion of DNA sequences from −576 to −412 and −412 to −305 resulted in decrease and increase of CAT activity, respectively, suggesting that there were positive and negative responsive elements in the SOD1 promoter (depicted in Fig. 2B as PRE and NRE). In Fig. 2A, Rb₂-specific induction was observed in every construct except the one that had minimal promoter (pRSP-55). Rb₂ induced SOD1 expression about 3-fold, regardless of its promoter strength, under the appropriate conditions. These results showed that the target sequence for Rb₂-specific induction was located in the −305 to −55 region. This region was the proximal part of the SOD1 gene promoter that consisted of a large number of transcription factor binding sites (10, 11). It is possible that induction of SOD1 gene by Rb₂ is mediated by activation of specific transcription factors.

**Increase of the AP2 Binding Activity by Ginsenoside Rb₂**—We therefore measured the effect of Rb₂ on the activation of transcription factors by MSA. We used double-stranded oligonucleotides corresponding to the possible binding sites of transcription factors including the metal binding factor, heat shock factor, Sp1, AP2, CCAAT-enhancer binding protein α, TATA box binding protein, and NF-κB binding sites in the SOD1 promoter. The HepG2 cells were incubated with or without Rb₂ in the media for 22 h and harvested for extracting nuclear protein. An equal amount (10 μg) of the nuclear protein of the control and Rb₂-treated HepG2 nuclear extract was done. Specific DNA-protein complex was observed (indicated by solid arrowhead). DNA fragment from −305 to −74 of the SOD1 promoter competed with AP2 oligonucleotide (lane 4). E, MSA using DNA fragment (−305 to −74) and purified AP2 protein. Specific complex was observed (indicated by filled arrowhead). F, MSA using AP2 oligonucleotide and purified AP2 protein. Note that the synthetic AP2 oligonucleotide and the SOD1 promoter region (−305 to −74) competed with each other. The location of free probe was indicated by blank arrowhead.
by Rb2 treatment. The binding of AP2 was further confirmed by the fact that the AP2-DNA complex was supershifted by the addition of anti-AP2 monoclonal antibody (Fig. 3A, lane 5). These results strongly indicated that there was a Rb2-specific increase in AP2 factor in a dose-dependent manner. The same result was also observed in MSA with Rb2-treated HeLa nuclear extract.

We tested whether Rb2 induction of AP2 was dependent on new RNA and protein synthesis. When actinomycin D, a transcriptional inhibitor, or cyclohexamide, a protein synthesis inhibitor, was treated for 1 h before Rb2 treatment, the AP2-DNA complex did not appear (Fig. 3C). These results indicate that the induction of AP2 by Rb2 treatment depends on the synthesis of new RNA and protein. The amount of AP2 protein was determined by Western blot analysis. Using antibody against an AP2 protein, we measured AP2 protein in nuclear extracts of untreated and Rb2-treated HepG2 cells. As shown in Fig. 3B, the AP2 protein was considerably increased from the steady state level by Rb2 treatment.

**Presence of AP2 Binding Sites in the Promoter Region of SOD1 Gene**—To determine whether the SOD1 gene promoter region contains an AP2 binding site, MSA using an oligonucleotide corresponding to the AP2 binding site was performed with Rb2-treated HepG2 nuclear protein. DNA fragments from the SOD1 promoter region were used in MSA as a competitor. A specific AP2 oligonucleotide-protein complex was observed (Fig. 3D, lane 1) and disappeared when the DNA fragment from −305 to −74 of the SOD1 promoter was added to the reaction mixture (Fig. 3D, lane 4). No competition was observed with other DNA fragments from the SOD1 promoter as competitors (Fig. 3D, lanes 2, 3, and 5). To further determine whether the transcription factor AP2 can bind to the proximal region of the SOD1 promoter, we performed MSA with purified AP2. Specific DNA-AP2 complexes were observed (Fig. 3E). The doublet formation of the shifted band indicated that there is more than one binding site (10). Labeled AP2 oligonucleotide was also put in competition with the DNA fragment of the SOD1 promoter reciprocally (Fig. 3F). These results suggested that functional AP2 binding sites were located in the proximal part (−305 to −74) of the SOD1 promoter region. This was also confirmed by DNA sequence analysis in which two sites of AP2 were located between −134 and −111 (−134CCGCCGCC−127 and −118CCCGCGG−111).

**The Transactivation of the SOD1 Gene by Rb2 through AP2 Binding Sites and Its Induction**—We demonstrated that AP2 factor was increased by Rb2 treatment, and its binding site was in the promoter region of the SOD1 gene. Naturally, we asked whether Rb2 promotes the activation of the SOD1 promoter, which has two binding sites of AP2. The results showed that Rb2 induced the CAT activity of SOD1 promoter-CAT constructs (Figs. 1 and 2). Almost identical results were also obtained from cells that were transfected with the pAP2w plasmid, which has two copies of AP2 binding sites linked to a herpes simplex virus-thymidine kinase promoter (Fig. 4A). The CAT activity of the plasmid pAP2m containing a mutant AP2 site was not affected by Rb2 (Fig. 4C). We also constructed a plasmid bearing mutant AP2 binding sites in the natural context. Mutated AP2 binding sites were introduced into pRSP-305 (see Fig. 4B). Two sites of AP2 were changed to mutant sequences (from −134CCGCCGCC−127 to −134CCATATCC−127, from −118CCCGCGG−111 to −118CCATATGC−111). These mutations abolished binding activity with the AP2 protein in vitro. Rb2 did not affect the level of CAT activity of this mutant plasmid, pRSP-305AP2m, whereas the CAT activity of the wild-type pRSP-305 increased about 5-fold (Fig. 4D). These findings strongly confirmed that the Rb2 transactivation of the SOD1 gene was mediated through AP2 sites and its induction.

**DISCUSSION**

In this study, we demonstrated that the activation of the SOD1 gene by ginsenoside Rb2 was mediated by the transcription factor AP2. Rb2 is present in relatively large quantities in the saponin fraction of _P. ginseng_. It has been reported that Rb2 increased RNA polymerase I and II activity (26) and inhibited tumor angiogenesis and metastasis (13). Rb2 is a dammarane-type saponin that has been shown to possess various biological activities such as a protein anabolic effect, an anti-diabetes effect, an anti-hyperlipemia effect, and an anti-inflammatory activity (27). Both Rb1 and Rb2 have the basic structure of 20(S)-protopanaxadiol. Rb1 has four molecules of glucose in the sugar moiety, whereas Rb2 has three molecules of glucose and one molecule of arabinose (Fig. 1A). Although there is only a minor difference in the sugar moiety, it has been suggested that the action mechanism of the two agents is somewhat different (26). In our study, it appeared that Rb2 was a more potent activator of the SOD1 gene than Rb1. As a result, in Fig. 1B PT seemed to somewhat repress the CAT activity, and total saponin did not affect the CAT activity. There is an antagonistic action in the ginseng saponin components. Ginsenosides can stimulate cell growth and inhibit cell proliferation (28, 29). Ginsenoside Rg1 enhances the conversion of arginine to citruline but not Rb1 (30). The process of Rb2 induction appears not to be through the oxidoreduction pathway but rather through the transcription factor AP2. The finding of AP2 binding sites on the SOD1 promoter suggests the importance of SOD1 in the process whereby this transcription factor is activated. Transcriptional activation by AP2 involves the 52-kDa AP2 protein binding to a specific DNA motif found in the cis-regulatory region of the gene (31). AP2 activity is regulated in a cell

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2 Y. H. Kim and H. M. Rho, unpublished results.
type-specific manner (32) and is induced by phorbol esters, retinoic acids, and cAMP (31–33). Additionally, mRNA levels of AP2 have been shown to increase dramatically upon differentiation, indicating that the expression of AP2 is regulated during differentiation (33). The promoter of the AP2 gene has a functional AP2 site, and a positive autoregulatory loop has been detected in this promoter (34). AP2 also has a crucial role in the induction of the antioxidant enzyme heme oxygenase 1 by heme (35). These observations suggest that common induction mechanisms exist in the cells for antioxidant enzymes. We interpret these results to mean that Rb2 can accelerate autoregulation of transcription factor AP2 and that increased AP2 elevates the cellular amount of antioxidant enzymes such as SOD1. These results showed us a novel action mechanism of ginseng saponin on SOD1 transcription and could also provide a molecular link between ginseng saponin intake and its inhibitory effects on aging and mutation by radical oxygen.

Acknowledgments—We thank T. Williams of Yale University for the kind gift of SE4, a monoclonal antibody against AP2.

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J. Biol. Chem. 1996, 271:24539-24543.
doi: 10.1074/jbc.271.40.24539

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