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Xenobiotic-Responsive Element for the Transcriptional Activation of the Rat Cu/Zn Superoxide Dismutase Gene

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Cu/Zn superoxide dismutase (*SOD1*) catalyzes the dismutation of superoxide radicals produced from biological oxidation and environmental stresses. A number of xenobiotics are toxic because they generate free radicals, such as superoxide and hydroxyl radicals, through a redox cycle. The xenobiotic responsive element (XRE) was located between the nt -268 and -262 region of the 5'-flanking sequence of the *SOD1* gene. Functional analyses of this element by deletion, mutations, and heterologous promoter systems confirmed that the expression of the *SOD1* gene was induced by a xenobiotic through the XRE. Gel mobility shift assays showed the xenobiotic inducible binding of the receptor-ligand complex to XRE. The cytoplasmic fraction from nontreated HepG2 cells also contains the factor as a cryptic form and prominently reveals its DNA-binding activity by incubation with βNF *in vitro*. These results suggest that the XRE participates in the induction of the rat *SOD1* gene by xenobiotics.

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 the oxygen and hydrogen peroxide (1). SOD not only prevents the Fenton reaction and DNA nicking *in vitro*, but it also protects against toxicity by H_2O_2 *in vivo* without O_2^- being directly involved in the generation of DNA damage (2). The production and/or removal of superoxides has been observed to play significant roles in a variety of critical homeostatic mechanisms both at the cellular and the organismic levels. Since the biological macromolecules are targets for the damaging action of the abundant oxygen radicals, it is assumed that these increased superoxides should be eliminated by SOD1. Therefore, the regulation and induction mechanism of the *SOD1* gene would be of great interest (3). It has also been reported that SOD1 could prevent oncogenesis and tumor promotion (4); reduce the cytotoxic and cardiotoxic effects of anticancer drugs (5); protect against reperfusion damage of ischemic tissue (6). A recent report suggested that overexpression of SOD1 and catalase could increase the average lifespan of the fly (7).

A number of xenobiotics are toxic because they generate free radicals such as superoxide and hydroxyl radicals through a redox cycle (8). Polychlorinated aromatic hydorcarbons such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and polycyclic aromatic hydrocarbons are effective transcription inducers of several genes, including cytochromes P-450 (CYP1A1 and CYP1A2) (9, 10), NAD(P)H:quinone oxidoreductase (11, 12), glutathione S-transferase Ya subunit (13) and UGT1s (UGT1A1 and UGT1A2) (14). These inducers bind to aromatic hydrocarbon (Ah) receptors in the cytoplasm, and the Ah receptor-ligand complex translocates into the nucleus, depending upon the presence of the Ah receptor nuclear translocator. The nuclear-localized Ah receptor-ligand complex interacts with a specific *cis*-acting DNA sequence, termed the xenobiotic-responsive element (XRE), and it stimulates the transcription of these genes (15, 16). From the sequence analysis of the transcription factor binding sites, the XRE sequence was located between −268 and −262 of the 5′-flanking sequence of the *SOD1* gene (17).

In this study, the induction of the *SOD1* gene by xenobiotics, β-naphthoflavone (βNF), t-butylhydroquinone (tBHQ) and iodoacetamide (IA) was investigated by transient transfection of the rat *SOD1* promoter-CAT constructs. The deletion, mutation, and heterologous promoter systems were used to study the function of the XRE. A specific interaction of the XRE with the nuclear-

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Abbreviations used: SOD1, Cu/Zn superoxide dismutase; *SOD1*, Cu/Zn superoxide dismutase gene; XRE, xenobiotic-responsive element; Ah receptor, aromatic hydrocarbon receptor; CAT, chloramphenicol acetyl transferase; *tk*, thymidine kinase gene; βNF, β-naphthoflavone; tBHQ, *t*-butylhydroquinone; IA, iodoacetamide; MSA, mobility shift assays; nt, nucleotide; PBS, phosphate-buffered saline.

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localized Ah receptor-ligand complex was also investigated by gel mobility shift assays.

MATERIALS AND METHODS

Plasmid constructions. The 1.7 kb *Bam*HI/*Sma*I fragment (nt −1633 to +85) from the rat *SOD1* gene (17) was inserted into the pBLCAT3 (18). Unidirectional 5′ deletion mutants were produced by cutting 5′ region of the *SOD1* promoter with *Sph*I and *Bam*HI, followed by subsequent treatment with exonuclease III (19). The endpoints of deletion were confirmed by DNA sequencing with a Sequenase kit (US Biochemicals). For the construction of pXREtk, the oligonucleotide of the XRE sequence, which was corresponding to −268 to −262 of the *SOD1* promoter, cloned into the *Bam*HI site of pBLCAT2Δ, which is derived from pBLCAT2 (18). The plasmid pBLCAT2Δ has a minimal region (−80 to +51) of the herpes simplex virus thymidine kinase promoter. Three copies of the XRE oligonucleotide were introduced. The plasmid pmXREtk is a mutant of pXREtk with three copies of the mutated XRE site. Their sequence follows: SOD-XRE, 5′-GATCAGGCGCGCACGCAGGCCTCG-3′/3′-TCCGCGCGTGCGTCC GGAGCCTAG-5′; SOD-mXRE, 5′-GATCAGGCGCGAAATAAGGCCTCG-3′/3′-TCCGCGCTTTATTCCGGAGCCTAG-5′. The insertion of the XRE consensus and mutant sequences was confirmed by DNA sequencing.

Cell culture and transfection. Human HepG2 hepatoma cells were grown in Dulbecco's modified Eagle's medium (DMEM)/10% fetal calf serum/ penicillin G sodium at 100 units per ml/ streptomycin sulfate at 100 μg per ml/ amphotericin B 250 ng per ml. Cells were seeded into 60 mm plastic culture dishes (30–50% confluence) for 24 h prior to transfection. An equal amount (3.0 pmole) of the various constructs was transfected to the cells by the calcium phosphate DNA coprecipitation method (20). Five μg of pRSVβ-gal plasmid (21) was introduced in all experiments to correct the variations of transfection efficiency.

Chemical treatment and nuclear extract preparation. Chemicals were added to the culture medium at 36 h after transfection, and the cells were maintained for an additional 22 h. The various chemicals were evaluated for cytotoxicity over a concentration range of 1–500 μM by monitoring cell death. Nuclear extracts were prepared by a modified procedure of Andrews and Faller (22).

CAT assay. A CAT assay was performed as described previously (23). 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 7.9) 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 (24). Equal quantities of proteins were assayed for CAT activity on the basis of β-galactosidase activity. Extracts were incubated with 0.025 μCi of [^{14}C] chloramphenicol/ 0.25M Tris-Cl pH7.6/ 0.4 mM acetyl coenzyme A for 1 h at 37°C. The enzyme assay was terminated by adding ethylacetate. The organic layer was analyzed by TLC with chloroform/methanol (95:5). After autoradiography, both acetylated and unacetylated forms of [^{14}C] chloramphenicol were scraped from the plate, and the conversion of chloramphenicol to the acetylated form was calculated by measuring radioactivities. The relative CAT activities were calculated from the percent conversion. The results are the average of three independent experiments.

Mobility shift assay. The synthetic oligonucleotides for the XRE site were purchased from the Genosys Co. The double-stranded oligonucleotide was labeled with [γ-^{32}P]ATP and polynucleotide kinase (24). An equal amount (10 μg) of nuclear extract from each sample was mixed with labeled oligonucleotide for 20 min at 20°C in a 15 μl solution containing 10 mM HEPES, 100 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 10% glycerol, and 2 μg of poly[dI-dC]. The binding reaction mixtures were electrophoresed in 6% acrylamide gels in 0.5x TBE (44 mM Tris, 44 mM boric acid, and 1 mM EDTA). After electrophoresis, gels were dried and exposed to X-ray film. For competition assays, the binding reaction was performed with excess of cold probe or competitor DNA.

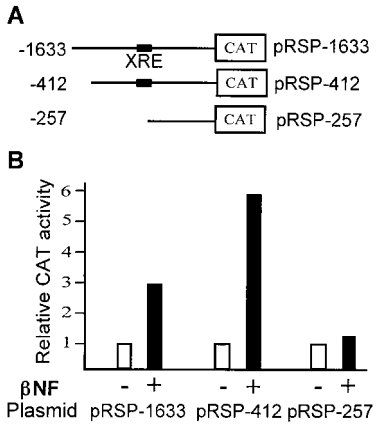

FIG. 1. Schematic diagram of SOD-CAT plasmids and the effect of βNF on the induction of the *SOD1* gene. (A) Schematic diagram of the *SOD1* promoter attached to CAT (chloramphenicol acetyltransferase). The numbers in the names of the plasmids represent the deletion points of the *SOD1* 5′-flanking sequence. The pRSP-1633 and pRSP-412 contain the XRE site, but pRSP-257 does not. (B) Activation of the *SOD1* promoter by βNF. The transfected cell was treated with βNF (50 μM) for 24 h. Relative CAT activities with (+) and without (−) βNF were indicated by the solid bar and open bar, respectively.

RESULTS AND DISCUSSION

Induction of the SOD1 gene by xenobiotics. The 1.7 kb DNA fragment of the 5′-flanking region (nt −1633 to +85) from the rat *SOD1* gene (17) was inserted into the pBLCAT3. Serial deletions from −1633 to −412 and to −257 were performed (Fig. 1A). These plasmids were designated pRSP-1633, pRSP-412 and pRSP-257, respectively. The plasmids pRSP-1633 and pRSP-412 contain a putative XRE sequence, but pRSP-257 does not (Fig. 1A). The complementary sequence of the XRE was located between −268 and −262 of the *SOD1* gene. The DNA recognition sites for the dioxin-Ah receptor complex termed XRE (xenobiotic responsive element) was previously identified (25). The dioxin-Ah receptor complex binds to DNA containing the core sequence TWGCGTG. The induction and activation mechanism of the *SOD1* gene by a xenobiotic was investigated as follows. These constructs were transfected into HepG2 cells and transient expression of the CAT enzyme driven by the deleted sequences was determined in the absence or presence of β-naphthoflavone (βNF) (50 μM). At thirty-six hours after transfection, βNF was added to the transfected cells at 50 μM of concentration. After 22 h, the CAT activity of each transfected cell was determined. The three- and six-fold induction of CAT activity by βNF was observed with pRSP-1633

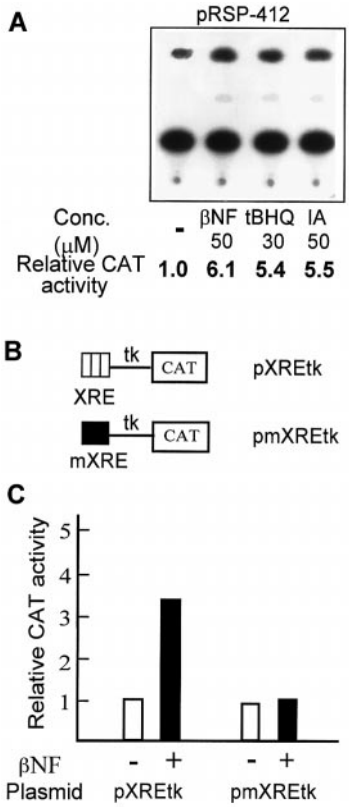

FIG. 2. The effect of xenobiotics on the *SOD1* gene and functional analysis of XRE with mutation and heterologous promoter system. (A) HepG2 cells transfected with plasmid pRSP-412 were treated with various xenobiotics: *β*-naphthoflavone (*β*NF), *t*-butylhydroquinone (tBHQ), and iodoacetamide (IA) in the indicated amounts. Relative CAT activity is expressed as compared with the CAT activity obtained in untreated cells. (B) Schematic diagram of synthetic XRE (pXREtk) and mutated XRE (pmXREtk) in the heterologous promoter (*tk*) attached to CAT. Three copies of the XRE oligonucleotide were introduced into the plasmid pXREtk. The plasmid pmXREtk is a mutant of pXREtk with three copies of mutated XRE site. (C) Effect of *β*NF on the synthetic XRE and mutated XRE in the heterologous promoter. Relative CAT activities with (+) and without (−) *β*NF (50 *μ*M) are indicated by the solid bar and open bar, respectively.

and pRSP-412, respectively, identifying a region that contains XRE. In contrast, pRSP-257 failed to respond to *β*NF (Fig. 1B). Relative CAT activity was expressed as compared with CAT activity obtained in untreated cells. To determine whether XRE is responsible for the induction of other xenobiotics, cells transfected with pRSP-412 were treated with various doses of t-butylhydroquinone (tBHQ) and iodoacetamide (IA) (Fig. 2A). The CAT activity of cells transfected with pRSP-412 was also activated about five-fold by treatment of tBHQ and IA (Fig. 2A). When HepG2 cells were transfected with pRSP-412, basal level CAT activity was shown due to the existence of the negative regulatory element (NRE) (3).

A number of xenobiotics are toxic because they reduce molecular oxygen by one electron to generate superoxide through a redox cycle (8). The oxidation of substances, such as a low-density lipoprotein, is generally protected by a complex antioxidation system involving superoxide dismutase, glutathione and glutathione peroxidase, Vitamin E, ascorbic acid, and so on. The induction of the *SOD1* gene would be very advantageous when the metabolism of specific chemicals produces the superoxide.

Identification of a cis-acting element required for the induction by βNF. We demonstrated that the *SOD1* gene was induced by *β*NF. To determine whether *β*NF promotes the activation of the *SOD1* gene through the XRE, the heterologous-promoter system was used. Plasmid pXREtk has three copies of the double-stranded oligonucleotide corresponding to the SOD-XRE linked to a herpes simplex virus-thymidine kinase promoter (*tk*) connected to the CAT gene (Fig. 2B). When this plasmid pXREtk was transfected into the HepG2 cells, four-fold induction of CAT activity by *β*NF was observed. But the CAT activity of the plasmid pmXREtk containing a mutant XRE was not affected by *β*NF (Fig. 2C). These findings strongly confirmed that the induction of the *SOD1* gene by xenobiotic was mediated through the XRE site.

Detection of XRE binding protein (Ah receptor) and its localization. To detect factors that interact with the XRE sequence, gel mobility shift assays were carried out by using a double-stranded synthetic oligonucleotide, as corresponding to XRE as a probe to assess whether the nuclear-localized Ah receptor-ligand complex interacted with the XRE of the *SOD1* gene (Fig. 3). When a nuclear extract prepared from untreated HepG2 cells was incubated with a ^{32}P-labeled SOD-

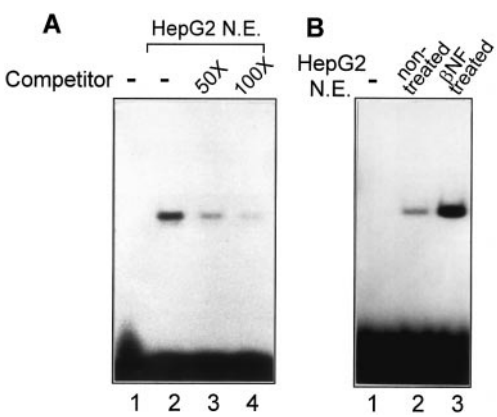

FIG. 3. Binding and induction of the XRE binding protein *in vivo.* The gel mobility shift assay was performed using nuclear extracts (N.E.) prepared from untreated HepG2 cells (A) and *β*NF treated HepG2 cells (B). The probe corresponding to XRE was labeled at its 5′-end. The intensity of the retarded DNA-protein complex was increased when the cells were treated with *β*NF (B, lane 3).

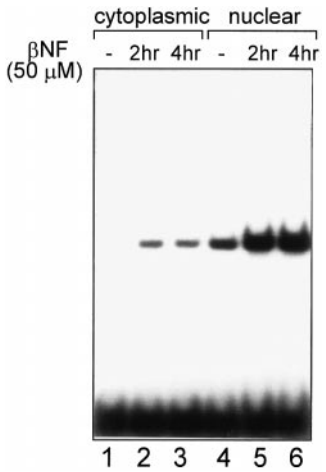

FIG. 4. Induction of XRE binding protein *in vitro.* The gel mobility shift assay was performed using cytoplasmic fractions and nuclear extracts prepared from HepG2 cells grown in the absence of βNF. The reaction mixture was incubated with 50 μM βNF (lanes 2, 3, 5, and 6) *in vitro.* The intensity of retarded DNA-protein complex was increased when the reaction mixture was incubated with 50 μM βNF (lanes 5 and 6).

XRE probe, one DNA-protein complex was observed (Fig. 3A). The amount of a shifted band was completely diminished by adding excess amounts (50×, 100×) of unlabeled SOD-XRE oligonucleotides, indicating the specificity of this protein-DNA interaction (Fig. 3A). When using a nuclear extract from βNF-treated cells, the intensity of the DNA-protein complex was increased (Fig. 3B). These results indicated that the *SOD1* gene is induced by xenobiotic through the binding of the Ah receptor to the XRE site of the *SOD1* gene.

The induction of the XRE binding factor was also investigated in the cell-free system. The cytoplasmic fraction or nuclear extract prepared from nontreated HepG2 cells was incubated with βNF (50 μM) for the indicated time to examine the generation of their binding activity to the XRE. The incubation of nuclear extract with βNF gave the strong intensity of retarded band (Fig. 4). Although the XRE-binding activity was absent in the nontreated cytoplasmic fraction, the incubation of the cytoplasmic fraction with βNF resulted in the induction of the factor that gave the retarded band of the XRE with the same mobility as the nuclear factor (Fig. 4, lane 1–3). The time of βNF necessary for a maximal level of the induction was 2 h (Fig. 4). Thus, the results show that βNF induces the XRE-binding activity in the preexisting cryptic factor.

In this study, the rat *SOD1* gene was induced by xenobiotics, such as βNF, tBHQ and IA. This induction mechanism may be mediated through the xenobiotics response element (XRE). Using the gel mobility shift assay, we have demonstrated the presence of a factor that specifically interacted with the XRE. It has been shown that the Ah receptor, when complexed with polycyclic ligands, is a DNA binding protein that interacts specifically with the Ah response elements of the cytochrome P450 IA1 gene (25). Saatcioglu *et al.* (26) reported two nuclear factors, in addition to the Ah receptor, which also interacts with the XREs of the cytochrome P450IA1 gene. Both factors, called XF1 and XF2, interact sequence specifically with XREs. Another xenobiotic-responsive element was identified and named antioxidant responsive element (ARE), which was required for transcriptional activation by phenolic antioxidants and a metabolizable planar aromatic compound (27).

Several models can be considered for the mechanism of the activation of the cryptic DNA-binding factor by a xenobiotic. The well-known model is that the xenobiotic receptor transforms itself to an XRE-binding protein by ligand binding like steroid hormone action (28). Alternatively, the activation of the DNA-binding ability of the receptor requires the association or the dissociation of a cofactor that is triggered by ligand binding. However, the molecular mechanism of activation of these receptors by ligand binding remains to be clarified.

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